Cloning, Reconstruction and Heterologous Expression of Secondary Metabolite Gene Clusters from Fusarium

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BY
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AALBORG UNIVERSITY
DENMARK

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Preface

The present thesis was submitted as part of the requirements for attaining the PhD degree at the faculty of Engineering and Science, Aalborg University. The thesis is based on work carried out in the period from February 2016 to January 2019. The research project was fully supported by Novo Nordisk Foundation grant NNF15OC0016028

I have been enrolled at the Department of Chemistry and Bioscience, Faculty of Engineering and Science during this project. The research project was carried out at Aalborg University, Esbjerg, Denmark. As a part of the study I travelled four months abroad to the laboratory of Dr. Donald Gardiner, CSIRO, Brisbane, Australia.

The photograph appearing on the cover page are *Fusarium solani* transformants expressing secondary metabolites from this study.

The thesis is based on the following papers:

1. **Nielsen, M. R**., Sondergaard, T.E., Giese, H., Sørensen, J.L. (2019) *Advances in linking polyketides and non-ribosomal peptides to their biosynthetic gene clusters in Fusarium*.
   Manuscript submitted to Current Genetics, Springer.
2. **Nielsen, M. R.,** Holzwarth, A. K. R., Kastaniegaard, K., Sondergaard, T.E., Sørensen, J.L. (2019) *A new vector system for ectopic gene expression in the crop pathogen Fusarium solani*.
   Manuscript submitted to Journal of Fungi, MDPI
3. **Nielsen, M. R.**, Wollenberg, R. D., Westphal, K. R., Sondergaard, T. E., Wimmer, R., Gardiner, D. M., Sørensen, J. L. (2019) *Heterologous Expression of intact Biosynthetic Gene Clusters in Fusarium graminearum*.
   Manuscript submitted to Fungal Genetics & Biology, Elsevier.
4. **Nielsen, M. R.**, Pedersen, T. B., Holzwarth, A. K. R., Perez, T., Westphal, K., Wimmer, R., Sondergaard, T. E., Sørensen, J. L. (2019) *The final piece to the polyketide pigmentation puzzle in Fusarium solani*.
   Manuscript in draft

Furthermore, I contributed to the following publications as side-projects. These publications are not part of the focus of this thesis but are included to demonstrate the worth of the methods presented:

1. Wollenberg, R. D., Sondergaard, T. E., **Nielsen, M. R.**, Knutsson, S., Pedersen, T. B., Westphal, K., Wimmer, R., Gardiner, D. M., Sørensen, J. L. (2019) *There it is! Fusarium pseudograminearum did not lose the fusaristatin gene cluster after all*.
   Fungal Biology, doi: 10.1016/j.funbio.2018.10.004
2. Sørensen, J. L., Benfield, A. H., Wollenberg, R. D., Westphal, K., Wimmer, R., **Nielsen, M. R.**, Nielsen, K. F., Carere, J., Covarelli, L., Beccari, G., Powell, J., Yamashino, T., Kogler, H., Sondergaard, T. E., Gardiner, D. M. (2018) *The cereal pathogen Fusarium pseudograminearum produces a new class of active cytokinins during infection*.
   Molecular Plant Pathology, doi: 10.1111/mpp.12593
3. Blum, A., Benfield, A. H., Sørensen, J. L., **Nielsen, M. R.**, Bachleitner, S., Studt, L., Beccari, G., Covarelli, L., Batley, J., Gardiner, D. M. (2019) *Regulation of a novel Fusarium cytokinin in Fusarium pseudograminearum*.
   Fungal Biology, doi: 10.1016/j.funbio.2018.12.009

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First and foremost I owe my most sincere thanks to my project supervisor, Associate Professor Jens Laurids Sørensen, for his invaluable support and guidance. Thank you for providing a positive outlook and for inspiring me to pursue an academic career.

I also want to thank Dr. Donald Max Gardiner for welcoming me into his laboratory and for introducing me to methods and tricks critical to the success of my project, and Professor Reinhard Wimmer for taking time teaching me to operate the chemical analysis equipment.

My sincerest thanks go to Ailisa Blum, Anca Rusu and Rasmus Dam Wollenberg for answering my never-ending questions about PCR, cloning, transformation and sequencing. Indeed, you showed me just how efficient experiments can be performed in the laboratory.

I would like to thank all my colleagues in Aalborg, especially Henriette Giese, Teis E. Sondergaard and Klaus R. Westphal for feedback, discussions and inspiration. A huge thank you goes out to my co-students Mahdi, Hülya, Kasia, Tobias, Sebastian and Trine for the countless coffee breaks and Friday afternoon beers. Thank you, Jens M., Heidi, Dorte, Linda and everyone at the department for creating a friendly and positive working atmosphere at AAU Esbjerg.

Last but not least, I would like to express my appreciation and thanks to my family and friends for their enormous support and encouragement throughout the project. You never doubted my dedication and boosted my confidence when needed most.

Mikkel Rank Nielsen

February 2019

Esbjerg, Denmark

English summary

Filamentous fungi are capable of producing a vast array of secondary metabolites evolved to secure biological niches, deter predators, or combat other microorganisms. The biochemical potential of filamentous fungi is considered to pose as a rich and untapped resource of unique molecules with different bioactivities. In the recent decades, a research focus has been to describe the molecular products of these microorganisms which have included toxins and virulence factors, and drug leads such as antibiotics. The secondary metabolism of filamentous fungi such as the ascomycete *Fusarium* spp. is therefore considered of relevant to society from a health and biotechnological point of interest. The majority of *Fusarium* metabolites are currently unexplored as the cultivation conditions triggering the formation of secondary metabolites are impossible to predict and difficult to mimic in the laboratory.

In this study, I set out to advance available molecular genetic approaches in order to unlock and characterize novel *Fusarium* metabolites. A literature review presents the status of currently described biosynthetic pathways and products and provides a thorough walkthrough of the many relevant methods and techniques available. The fraction of metabolites that are produced under laboratory cultivation conditions pose as low hanging fruits in this setting. And in order to unravel the full metabolite potential of these fungi, genetic activation strategies must be taken into hand. *F. solani* is a species complex comprising several uncharacterized polyketide synthase and non-ribosomal peptide synthetase genes. An overexpression vector system was developed to enable targeted activation of silent biosynthetic genes. Transformants overexpressing transcription factors enabled activation of silent polyketide biosynthesis pathways including those producing aromatic compounds involved in mycelial and perithecial pigmentation.

The focus of this project was to adapt the concept of heterologous expression to achieve activation of biosynthetic pathways that are inactive in their native host. The application of yeast recombination cloning was used to assemble large plasmids containing intact biosynthetic gene clusters. The major challenges in this project were the purification of gene cluster containing plasmids and introducing them into a filamentous fungal host. An outcome of this project is thus my experience applying such techniques. Secondary metabolite pathways were cloned, reconstructed in yeast, introduced and heterologously expressed in the well-described *F. graminearum* strain PH-1. *F. graminearum* transformants expressing the *Fusarium* cytokinin gene cluster from *F. pseudograminearum* was applied to

solidify the connection between this novel class of active compounds and the underlying genetic material. Furthermore, these transformants were applied in plant infection assays to assess the contribution of these metabolites to pathogenicity.

To push the envelope of heterologous expression, a gene cluster was chosen at twice the size of what has previously been heterologously expressed in a filamentous fungal host. The 54 Kbp gene cluster responsible for the formation of lipopeptides in *F. pseudograminearum* (*PKS40 + NRPS32*), was successfully introduced and heterologously expressed in *F. graminearum*. Wild type product titers were observed in transformants. This example demonstrates the power of yeast recombination as a tool for cloning large and functional gene clusters. Lastly, I wanted to work with a proof-of-concept study where an uncharacterized and silent biosynthetic pathway was activated via heterologous expression. For many years, it has been known that the *PKS35* is responsible for the perithecial pigmentation of *F. solani*, although no compounds have been isolated and associated with these genes. To investigate this particular pathway, we used targeted activation in *F. solani* together with heterologous expression in *F. graminearum* and *S. cerevisiae*. This mixed methods strategy enabled formation and observation of compounds not produced in the *F. solani* progenitor strain, demonstrating heterologous expression as a gateway to achieve activation of silent biosynthetic genes. Future endeavors concerning secondary metabolism of *Fusarium* will benefit from applying the presented methods to assemble and transform intact and functional gene clusters. *F. graminearum* is an ideal choice of expression host and it has a vast arsenal of associated transformation protocols and available vector systems.

Bioinformatic prediction services are commonly applied to predict the metabolite products of gene clusters based on sequence similarity to characterized orthologues. However, in many cases remains the predicted metabolite to be isolated from a *Fusarium* isolate and there is still need for experimental evidence to solidify the functionality of many biosynthetic genes. The content of this thesis gives a current overview of polyketide and non-ribosomal product discovery from *Fusarium* spp. and present the application of relevant strategies from the molecular genetics toolbox.

Dansk resumé

Filamentiøse skimmelsvampe kan producere en bred pallette af sekundære metabolitter udviklet for at sikre biologiske nicher, afskrække rovdyr eller for at bekrige andre mikroorganismer. Skimmelsvampenes biokemiske potentiale opfattes som en rig og uudnyttet ressource af unikke molekyler med forskellige bioaktiviteter. De seneste årtier har et forskningsfokus været at beskrive de molekylærer produkter fra disse mikroorganismer, der har inkluderet toksiner, virulensfaktorer, og medicinalstoffer, som for eksempel antibiotika. Den sekundære metabolisme i filamentiøse skimmelsvampe så som sæksporesvampeslægten *Fusarium* er derfor fundet relevante for samfundet set fra et sundhedsmæssigt og bioteknologisk perspektiv. Størstedelen af *Fusarium* slægtens metabolitter er for nuværende ikke opdagede, da de specifikke vækstbetingelser, der forårsager dannelsen af sekundære metabolitter, er umulige at forudsige og kan være svære at efterligne i laboratoriet.

I dette studie vil jeg gerne videreudvikle de eksisterende molekylære genetiske værktøjer for at kunne aktivere og karakterisere nye metabolitter fra *Fusarium*. Et litteratur review præsenterer status af nuværende beskrevne biosyntetiske reaktionsveje og produkter, samt giver en grundig gennemgang af de mange tilgængelige relevante metoder og teknikker. Den fraktion af metabolitterne, der er produceret under laboratorie dyrkningsbetingelser, betragtes som lavt hængende frugter i denne sammenhæng. For at fremkalde det fulde metaboliske potentiale af disse skimmelsvampe, må genetiske aktiveringsstrategier tages i brug. *F. solani* udgør et artskompleks, der bærer flere ikke karakteriserede polyketidsyntase og ikke-ribosomale peptidsyntetase gener. Et over-ekspressions vektorsystem blev udviklet for at muliggøre aktiveringen af tavse biosyntetiske gener. Transformanter som over-ekspresserede transkriptionsfaktorer muliggjorde aktiveringen af tavse biosyntetiske polyketidsyntese reaktionsveje, inklusive dem, der producerer aromatiske farvestoffer i mycelium og perithecium.

Fokus i dette projekt var at adaptere konceptet heterolog ekspression til at opnå aktivering af biosyntetiske reaktionsveje, der er tavse i deres naturlige vært. Anvendelsen af gær rekombinations-kloning blev brugt til at samle store plasmider indeholdende intakte biosyntetiske genklostre. Den største udfordring i dette projekt var oprensningen af et genkloster indeholdende plasmider og introduktionen af disse ind i en filamentiøs skimmelsvampsvært. Udbyttet af dette projekt er derfor min erfaring med anvendelse af sådanne teknikker. Sekundære metabolit reaktionsveje blev klonet, sammensat i gær, introduceret og heterologt ekspresseret i den velbeskrevne art *F. graminearum* stamme PH-1. *F. graminearum* transformanter, der udtrykte *Fusarium* cytokinin genklosteret fra *F. pseudograminearum*, blev brugt til at konsolidere forbindelsen mellem denne nye klasse af aktive stoffer og det

underliggende genetiske materiale. Ydermere blev transformanterne brugt i planteinfektionsforsøg for at undersøge disse metabolitters bidrag til patogenitet.

For at flytte grænser inden for heterolog ekspression blev et genkloster, dobbelt så stort som hvad der tidligere har været heterologt ekspresseret i en filamentiøs skimmelsvamp, udvalgt. Et 54 Kbp genkloster ansvarlig for formationen af lipopeptider i *F. pseudograminearum* (*PKS40* + *NRPS32*) blev med succes introduceret og heterologt ekspresseret i *F. graminearum*. Vildtype produktmængder blev observeret i transformanter. Dette eksempel demonstrerer kraften af gær rekombinations kloning som et værktøj til at samle store og funktionelle genklostre. Endelig ønskede jeg at arbejde med et proof-of-concept studie hvor en ikke karakteriseret og tavs biosyntetisk reaktionsvej blev aktiveret igennem heterolog ekspression. Igennem mange år har det været kendt, at *PKS35* er ansvarligt for pigmentering af *F. solani* perithecia, selvom ingen stoffer er blevet isoleret og forbundet med disse gener. For at undersøge denne specifikke biosyntese benyttede vi målrettet aktivering i *F. solani* sammen med heterolog ekspression i *F. graminearum* og i *S. cerevisiae*. Denne kombination af fremgangsmetoder muliggjorde formation og observation af stoffer, der ikke før er set produceret i den oprindelige *F. solani* stamme, hvilket demonstrerer at heterolog ekspression kan føre til aktivering af tavse biosyntetiske gener. Fremtidige studier omhandlende sekundære metabolitter fra *Fusarium* vil med fordel kunne benytte de præsenterede metoder til at samle og transformere intakte og funktionelle genklostre. *F. graminearum* er et oplagt valg som ekspressionsvært, og den har et kolossalt katalog af tilknyttede transformationsprotokoller og tilgængelige vektorsystemer.

Bioinformatiske analyseværktøjer benyttes ofte til at forudsige hvilke metaboliske produkter et genkloster kan producere baseret på sekvens ligheder til karakteriserede gener. Men i mange tilfælde mangler den forudsagte metabolit stadig at blive isoleret fra en *Fusarium* art og der er stadig brug for eksperimentel evidens for at konsolidere funktionaliteten af mange biosyntetiske gener. Indholdet i denne afhandling giver et aktuelt overblik over polyketid og ikke-ribosomale peptid produkt opdagelse fra arter af *Fusarium* slægten, og præsenterer anvendelsen af relevante strategier fra den molekylære genetiske værktøjskasse.

List of abbreviations

ATMT	*Agrobacterium tumefaciens*-mediated transformation
BGC	Biosynthetic gene cluster
bp	Base pair
DNA	Deoxyribonucleic acid
FAD	Flavin adenine dinucleotide
FCK	Fusarium cytokinin
f.sp	Forma speciales
HPLC	High pressure liquid chromatography
Kbp	Kilo base pair
KO	Knock-out
MS	Mass spectrometry
OE	Overexpression
OSMAC	One strain, many compounds
PMT	Protoplast-mediated transformation
R/L	Restriction and ligation
SM	Secondary metabolite
Sp.	Species
Spp.	Species (plural)
T-DNA	Transfer-DNA
USER	Uracil-specific excision reagent
PCR	Polymerase chain reaction

<u>Bioinformatic tools</u>

AntiSMASH	Antibiotics & Secondary Metabolite Analysis Shell
BLAST	The Basic Local Alignment Search Tool
CASSIS	Cluster Assignment by Islands of Sites
GO	Gene Ontology
NCBI	National Center for Biotechnology Information
SMURF	Seconday Metabolite Unique Regions Finder

<u>Enzymes and protein domains</u>

TC	Terpene cyclase
TF	Transcription factor
NRPS	Non-ribosomal peptide synthetase
A	Adenylation domain
T	Peptide acyl carrier domain
C	Condensation domain
PKS	Polyketide synthase
KS	β-Ketosynthase domain
AT	Acyl-transferase domain
ACP	Acyl-carrier protein domain

<u>Cultivation medium and chemical reagents</u>

ICI	Imperial Chemical Industries
PEG	Polyethylene glycol
PDA	Potato dextrose agar
YES	Yeast extract succrose
YPG	Yeast extract peptone glucose

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Aim

The overall aim of this thesis was to develop and apply workflow strategies to ensure expression of silent biosynthetic pathways from *Fusarium*. More specifically, the detailed aims were to:

- Develop a strategy for cloning large secondary metabolite gene clusters by yeast recombination. This includes adapting routine procedures such as yeast transformation, plasmid recovery and validation.
- Transformation of *Fusarium graminearum* for heterologous expression of foreign gene clusters. An additional goal was to achieve introduction and heterologous expression of large biosynthetic genes, such as non-ribosomal peptide synthetases.
- Application of targeted activation and heterologous expression methods to express and characterize novel polyketide compounds from *Fusarium solani*.

1. Introduction

The introduction is in part based on sections from the review article included in this thesis: A*dvances in linking polyketides and non-ribosomal peptides to their biosynthetic gene clusters in Fusarium* (**Paper 1**) by Nielsen, M. R., Sondergaard, T. E., Giese, H., Sørensen, J. L. (Submitted to Current Genetics, Springer).

1.1 Filamentous fungi and their secondary metabolites

In the recent decades there has been an enormous research focus on isolation and characterization of natural products from microorganisms such as filamentous fungi. Filamentous fungi exhibit a vast palette of molecules comprising both desirable drug leads and dangerous toxins (Bérdy 2005; Demain 2014). This arsenal of bioactive molecules has evolved in fungi contributing to fitness, such as deterring predators (Künzler 2018), combating other microorganisms or protecting against change in environmental conditions (Eisenman and Casadevall 2012). The unique and structural complexity of some compounds is not found elsewhere in nature, and can be difficult to mimic through chemical synthesis (Carlile et al. 2001; Hertweck 2009). Many fungal products are of major importance to humankind (Newman and Cragg 2012), especially the β-lactam and cephalosporin antibiotics, which in 2009 represented the most widespread applied antibiotics in the world (Hamad 2010). Other examples of developed pharmaceuticals are the antifungal agent griseofulvin, cholesterol-lowering lovastatin, or the ergot anti-migraine agents (Istvan 2001; Haarmann et al. 2009). Often, the natural products are used as scaffolds for semisynthetic derivatives broadening the potential of diversity of these molecules (Chun and Brinkmann 2011). Alternatively, the natural products have inspired fully synthetic products, such as the most sold fungicide azoxystrodin (Bartlett et al. 2002). In addition, filamentous fungi are capable of producing a range of detrimental compounds, such as mycotoxins which are monitored in food and feed. Infamous examples include the carcinogenic aflatoxins (Shotwell et al. 1966) and ochratoxins (Kuiper-Goodman and Scott 1989). Thus, the study of fungal biochemistry should be regarded as valuable to society, and be especially appealing from a biotechnological standpoint.

1.1.1 Fusarium

Soil-borne ascomycete fungi belonging to the genus *Fusarium* have high impact on health and agriculture (Nucci and Anaissie 2007; Dean et al. 2012). The genus is found in warm and temperate ecosystems throughout the globe often as plant pathogens contributing to major economic losses from infected crops (Mcmullen et al. 1997; Windels 2000; Michielse and Rep 2009). Many species are harmless, but species like *F. graminearum* (teleomorph *Gibberella zeae*) and *F. oxysporum* infect cereals and produce high amounts of mycotoxins rendering entire harvests unfit for consumption (Windels 2000). *Fusarium* head blight is considered one of the most serious crop diseases and is most commonly caused by *Fusarium graminearum* in Europe, America and Asia (Gilbert and Haber 2013). Naturally occurring outbreaks have affected human health as well as contributed to major financial losses (Nganje et al. 2004; O'Donnell et al. 2004).

Fusarium comprises more than 100-500 species (Leslie and Summerell 2006) capable of causing infection in plant, humans and domesticated animals (Summerell et al. 2010). The speciation of *Fusarium* has always posed a challenge to researchers due to the lack of distinguishing morphological features (**Figure 1**). Historically, the number of recognized species has varied between nine to >1000, depending on the identification scheme being implemented. Thus, the study of *Fusarium* is a genus comprising several species and metabolites of an importance that transcends science and agriculture (Leslie and Summerell 2006).

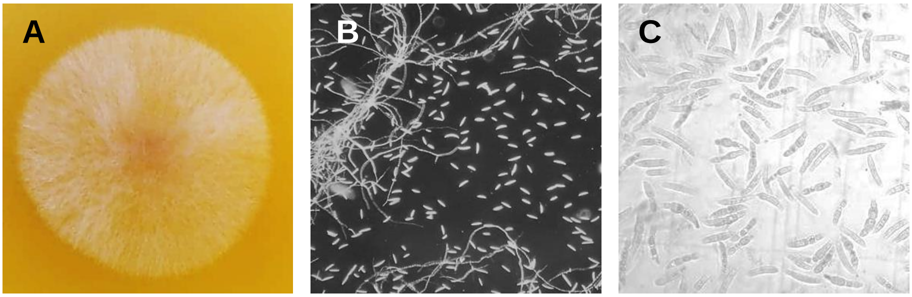

Figure 1 Morphological features of *Fusarium*. **A.** *F. graminearum* grows rapidly forming a dense white mycelium which will later turn yellow and red. **B.** Asexual sporulation in liquid culture. **C.** The morphology of macroconidia is a common identification characteristic for *Fusarium* species. *Fusarium* macroconidia appear as long needlelike cells containing several septa.

Like other eukaryotic ascomycetes *Fusarium* has the ability to produce small specialized compounds, secondary metabolites (SM) not associated directly with growth or reproduction, although hypothesized to contribute to fitness (Hoffmeister and Keller 2007; Brakhage 2013). In recent decades there has been a research focus on describing the biochemical and molecular machinery which controls the formation of these chemical compounds. *Fusarium* SMs exhibit an extreme diversity in function and chemical structures. They are usually formed by multi-domain core-synthases often cooperating with several decorating enzymes in a pathway to generate the final product. The genes encoding these enzymes are commonly found as neighbors to the core-synthetase gene and together they form a biosynthetic gene cluster (BGC) (Keller et al. 1997; Yu and Keller 2005). In addition genes encoding transcriptional regulators, transport proteins, and the odd product detoxification protein are found in the clusters. Unfortunately, many of these BGCs show little to no expression when grown under standard laboratory conditions (Gaffoor et al. 2005; Sieber et al. 2014) and therefore the potential undiscovered SMs are either not produced or present at levels too low to be detected by standard methods (Wiemann and Keller 2014). Although many molecules have been isolated and described (Hansen et al. 2015; Brown and Proctor 2016; Nielsen et al. 2019c), the full biochemical potential of the collected *Fusarium* secondary metabolome is yet to be explored.

1.2 Classes of secondary metabolites

SMs are biosynthesized from small precursor monomers like short chain carboxylic acids and amino acids from the primary metabolism. These precursors are polymerized by large synthase/synthetase enzymes like iterative polyketide synthases (PKS, type I and III), non-ribosomal peptide synthases (NRPS) or terpene cyclases (TC). *Fusarium* are capable of producing many terpenes (Brock et al. 2013; Burkhardt et al. 2016), some of which are important virulence factors such as trichothecines, nivalenol and deoxyvalenol (Marasas et al. 1979; Yoshida and Nakajima 2010) or plant hormones such as gibberellins (Bömke and Tudzynski 2009; Troncoso et al. 2010). However, the majority of characterized SMs belong in the chemical groups of polyketides (reduced and non-reduced), non-ribosomal peptides, or hybrid PKS-NRPS compounds (**Figure 2**) (Sieber et al. 2014; Hoogendoorn et al. 2018). Iterative Type I and III PKSs are large multi-domain enzymes that as a minimum contain β-ketosynthase (KS), acyl-transferase (AT) and acyl-carrier protein (ACP) domains which work together in an iterative cycle to elongate a polyketide chain with one ketide unit (McDaniel et al. 1994; Bentley and Bennett 1999). Fungal polyketides can exert great structural diversity. Generally the biosynthesis will start from an acetyl-CoA unit which is then elongated with malonyl-CoA units through Claisen condensation performed by the KS domain. However, in some cases the starter unit can stem from another PKS or a fatty acid synthase (Brown et al. 1996).

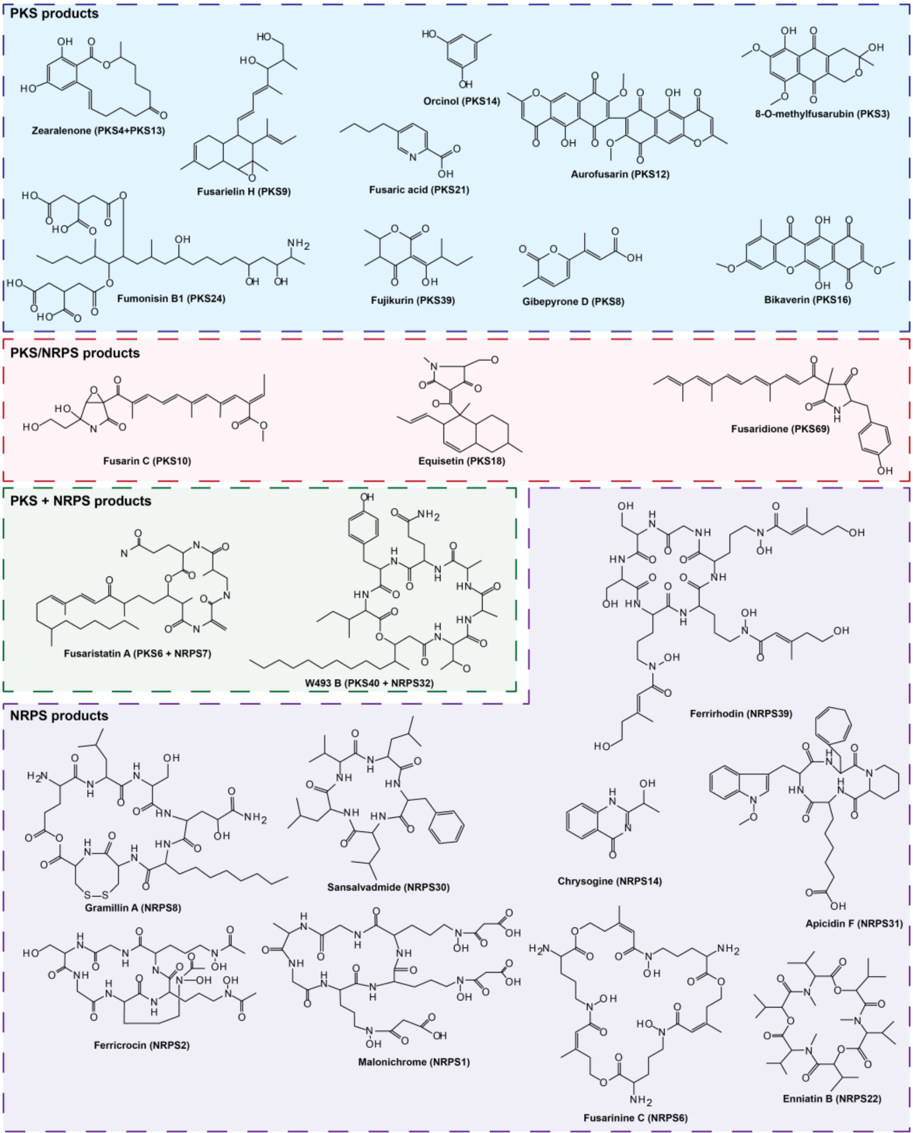

Figure 2 Chemical structures of polyketide, non-ribosomal peptide and hybrid products produced by species of *Fusarium*. Copied from **Paper 1** (Nielsen et al. 2019c).

In addition to the KS-AT-ACP module, PKSs may contain additional tailoring domains which add to the chemical diversity; e.g. reductase, dehydrogenase or methyltransferase domains (Meier and Burkart 2009). The PKS type I, which is most predominant in *Fusarium* (Brown and Proctor 2016), can be further subdivided into reducing or non-reducing PKSs yielding either fatty acid-like or true polyketide products, respectively. Lastly, the tailoring domains can skip an iteration as seen for zearalenone where only four out of five ketones are fully reduced (Gaffoor and Trail 2006). It is not surprising that prediction of the final product based on amino-acid sequence alone has proven to be impossible, also taking into account that similar PKSs may produce very distinct polyketide products.

NRPS are multi-modular assembly lines catalyzing the formation of small peptides from amino acid monomers. One NRPS module contains an adenylation (A), a peptide acyl carrier (T) and a condensation (C) domain. An NRPS is thus composed of one or more elongation modules (A-T-C) which catalyze the formation of a polypeptide chain. In addition, each module may contain tailoring domains e.g. epimerization or N-methylation domains, that contribute to the chemical diversity of non-ribosomal peptides (Finking and Marahiel 2004). The compound is then released from the synthetase by cyclization, reduction or hydrolysis, and peptide can be further modified by additional tailoring enzymes in the gene cluster such as cytochrome P450 monooxygenases and dehydrogenases.

At least 500 different NRPS substrates have been reported in filamentous fungi, which comprise non-proteinogenic amino acids, D– and-L forms, and even hydroxyl acids (Strieker et al. 2010). Some NRPSs incorporate fatty acyl chains leading to the formation of lipopeptides (Chooi and Tang 2010; Sørensen et al. 2014a). The A domain contains a binding pocket that recognizes a specific amino acid substrate (Conti et al. 1997), and substrate prediction algorithms was developed, first for bacterial NRPSs (Stachelhaus et al. 1999; Challis et al. 2000) and further modified to include eukaryotic NRPSs (Röttig et al. 2011; Khayatt et al. 2013; Knudsen et al. 2016). The feasibility of using these tools to predict *Fusarium* NRPS substrate accurately remains to be demonstrated (Wollenberg et al. 2017). In the case for both *NRPS* and *PKS* BGCs, the linking of biosynthetic metabolites to their respective genes is therefore dependent of experimental evidence.

1.3 Genomic resources

In order to understand the biosynthetic mechanisms behind secondary metabolism, knowledge of the genetic basis for SM biosynthesis is essential as it enables genetic manipulation and genome mining strategies. So far, genome sequencing has been carried out on 31 species representative of the *Fusarium* genus (Cuomo et al. 2007; Ma et al. 2010, 2014; Al-Reedy et al. 2012; Gardiner et al. 2012, 2014; Wiemann et al. 2013; Moolhuijzen et al. 2013; Lysøe et al. 2014; King et al. 2015; Vanheule et al. 2016; Brown and Proctor 2016) and the genomes reveal a potential for these fungi to produce more SMs than originally expected (Kroken et al. 2003; Sieber et al. 2014). Comparative analyses of biosynthetic genes reveal their distribution across the *Fusarium* metagenome which provides insight towards the evolution of BGCs and can guide efforts towards characterization of novel SMs (Ma et al. 2010). Available bioinformatic resources such as the FungiSMASH cluster prediction tool and the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository enable rapid detection of gene clusters in newly sequenced species (Blin et al. 2017; Epstein et al. 2018). Other tools such as SMURF and InterPRO enable functional prediction from protein sequences (Apweiler et al. 2000; Khaldi et al. 2010). Three recent studies have analyzed available *Fusarium* genomes for the presence of BGCs (Hansen et al. 2015; Brown and Proctor 2016; Hoogendoorn et al. 2018). Prediction of secondary metabolite gene clusters (and pseudo-genes) has been carried out and a numbering nomenclature was introduced (Hansen et al. 2012b, 2015). This has been maintained and has been expanded to provide a simple system by which to identify all the *PKS* and *NRPS* genes by a number (Brown and Proctor 2016). Some biosynthetic gene clusters are found in the majority of species of *Fusarium*, e.g. *PKS3*, *PKS7*, and *PKS8*, whilst others are restricted to a single phylogenetic clade e.g. *PKS29*, *30*, *31*, *32*, *33*, and *35* from the *F. solani* species complex. The distribution of *Fusarium* BGCs do not always follow a phylogenetic pattern and evidence for horizontal gene transfer events has been reported (Oide et al. 2006; Ma et al. 2010; Gardiner et al. 2012; Sieber et al. 2014). So far 67 *PKS* and 52 *NRPS* gene clusters have been identified distributed across the *Fusarium* metagenome. Only 16 out of 67 *PKS* and 11 out of 52 *NRPS Fusarium* genes been linked to their respective biosynthetic product (**Table 1**). Additionally, homology based prediction has assigned a handful of gene clusters to biosynthetic products isolated from other genera. Currently a handful of putative metabolites have been assigned to species of *Fusarium* based on gene orthology and synteny (Gaffoor et al. 2005; Hansen et al. 2012b, 2015; Wiemann et al. 2013; Brown and Proctor 2016; Hoogendoorn et al. 2018; Janevska and Tudzynski 2018). Although prediction tools have proven reliable, many of the predicted metabolites still remain to be detected in the fungal organism by chemical analyses. Gene comparisons may be useful in risk assessment as exemplified by the observation of a putative mycotoxin producing synthase in the genome of the biological control strain *F. oxysporum* Fo47 (Hoogendoorn et al. 2018). In short, creating the linkage between *PKS* and *NRPS* BGCs to their respective product can be supported by bioinformatic information. However, the conclusive evidence will for most cases be based on experimental results.

Table 1 Gene clusters, natural products and proposed activity of secondary metabolites isolated from species of *Fusarium*. Copied from **Paper 1** (Nielsen et al. 2019c).

Gene cluster	Product	Method[b]	Reference
PKS3	Fusarubins	Medium, KO	(Studt et al. 2012; Frandsen et al. 2016)
PKS4 + *PKS13*	Zearalenone	Split-marker, gene disruption	(Kim et al. 2005b; Gaffoor and Trail 2006; Lysøe et al. 2006)
PKS6 + *NRPS7*	Fusaristatin	KO	(Shiono et al. 2007; Sørensen et al. 2014b, a; Li et al. 2016)
PKS8	Gibepyrone	KO of PKS and TF	(Janevska et al. 2016; Westphal et al. 2018a)
PKS9	Fusarelins	OE-TF	(Sørensen et al. 2012a; Hemphill et al. 2017a)
PKS10	Fusarins	KO, Gene disruption	(Song et al. 2004; Brown et al. 2012)
PKS12	Aurofusarin	Gene disruption, Split-marker of PKS and TF	(Gaffoor et al. 2005; Kim et al. 2005a; Malz et al. 2005; Frandsen et al. 2006)
PKS14	Orcinol	OE-PKS	(Jørgensen et al. 2014)
PKS16	Bikaverin	KO	(Linnemannstöns et al. 2002; Wiemann et al. 2009; Sørensen et al. 2012b)
PKS17[a]	Depudecin	Homology	(Brown and Proctor 2016)
PKS18	Equisetin	OE of TF	(Kakule et al. 2015)
PKS21	Fusaric acid	Split-marker, OE-TF	(Brown et al. 2012; Niehaus et al. 2014b; Studt et al. 2016a)
PKS24	Fumonisins	KO	(Proctor et al. 1999, 2008)
PKS35	(*pigment*)	Gene disruption	(Graziani et al. 2004)
PKS39	Fujikurins	OE of PKS and TF	(Wiemann et al. 2013; Von Bargen et al. 2015)
PKS40 + *NRPS32*	W493	KO	(Nihei et al. 1998; Sørensen et al. 2014a)
PKS44[a]	solanapyrone	Homology	(Brown and Proctor 2016)
PKS45[a]	Tenellin	Homology	(Brown and Proctor 2016)
PKS51	(*virulence*)	OE-TF	(Niehaus et al. 2017a)
PKS52[a]	Alternapyrone	Homology	(Brown and Proctor 2016)
PKS54[a]	3-methylorsellinic acid	Homology	(Brown and Proctor 2016)
PKS55 + *PKS64*[a]	Oxononal benzaldehyde	Homology	(Brown and Proctor 2016)
PKS56[a]	Mellein	Homology	(Brown and Proctor 2016)
PKS69	Fusaridione	OE-PKS (plasmid)	(Kakule et al. 2013)
NRPS1	Malonichrome	Split-marker	(Oide et al. 2014)
NRPS2	Ferricrocin	KO, Split-marker	(Tobiasen et al. 2007; Oide et al. 2014)
NRPS4	(*hydrophobicity*)	OE-NRPS	(Hansen et al. 2012a)
NRPS6	Triacetylfusarinine	Split-marker	(Oide et al. 2006, 2014)
NRPS8	Gramillins	KO	(Bahadoor et al. 2018)
NRPS14	Chrysogine	OE-NRPS, KO	(Wollenberg et al. 2017)
NRPS22	Enniatin	Anti-serum screening and sequencing	(Haese et al. 1993; Liuzzi et al. 2017)
NRPS30	Sansalvamide	KO	(Romans-Fuertes et al. 2016)
NRPS31	Apicidins	OE-TF, KO	(Jin et al. 2010; Niehaus et al. 2014a)
NRPS39	Ferrirhodin	Heterologous expression	(Munawar et al. 2013)
NRPS42[a]	Hexadehydro-astechrome	Homology	(Hoogendoorn et al. 2018)
NRPS43[a]	Fumarylalanine	Homology	(Hansen et al. 2015)

[(a)]Metabolite assigned based on high nucleotide similarity. Nomenclature: polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), [(b)]Gene replacement /'Knock-out' by double-homologous cross-over (KO), Over-expression (OE), Transcription factor (TF).

1.3.1 Intraspecific variation

The quest for depicting the total metabolite palette of every species is complicated. In some cases, separate isolates of the same species do not share the same set of BGCs. A small fraction of isolates of *F. pseudograminearum* carry the fusaristatin A (*PKS6-NRPS7*) gene cluster, whilst isolates not producing fusaristatin A carry an unfunctional remnant of the cluster (**Figure 3**). Phylogenetic analysis of 99 Australian *F. pseudograminearum* strains reflected, to some extent, the ability to produce fusaristatin A. 15 out 99 strains, all originating from Western Australia were able to produce fusaristatin A. However, phylogenetic analysis could not support the grouping of producing strains in to a clade separate from the non-producers. These results suggest the loss of the gene cluster occurred relatively recently as a single evolutionary event (**Side project 1**) (Wollenberg et al. 2019). Nucleotide sequence analysis coupled with chemical analysis of isolates can in such cases contribute to mapping the metabolic potential of fungal strains. This case also underlines the importance of maintaining correct naming of filamentous fungal isolates and cultures.

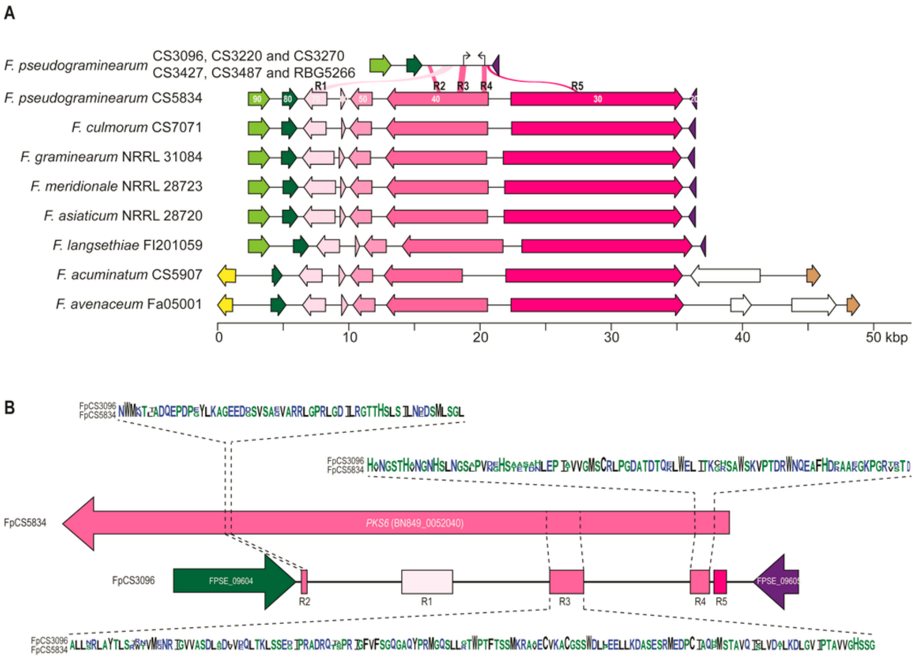

Figure 3 Comparative analysis of the fusaristatin gene cluster and remnant fragments in *Fusarium*. **A.** Illustration of the intact gene cluster in eight *Fusarium* species including the *F. pseudograminearum* strain CS5834. **B**. Predicted amino acid sequence of five remnant fragments occurring in non-producing strains including *F. pseudograminearum* CS3096. Copied from **Side project 1** (Wollenberg et al. 2019).

1.4 Regulation

The presence of a BGC does not directly imply the existence of a SM. The secondary metabolism of filamentous fungi is controlled by complex regulatory network of proteins responding to several environmental conditions such as available substrate, pH, light and temperature (**Figure 4**), as excellently reviewed by Axel A. Brakhage (Brakhage 2013). To no surprise, the vast majority of BGCs are unfunctional or silent in standard growing medium, and must be triggered to enable formation of the natural products. Additionally, any formed metabolites may be unstable, or present in undetectable quantities (Hansen et al. 2015).

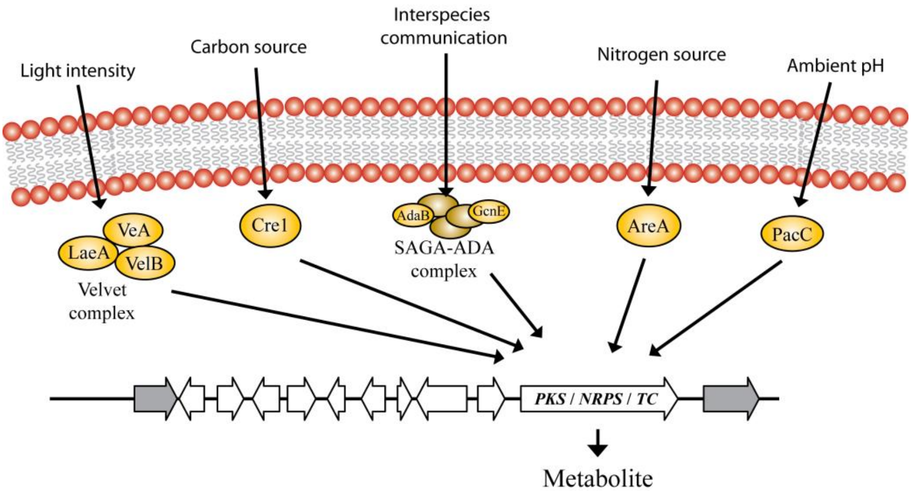

Figure 4 Extracellular environmental signals and physiochemical conditions induce activation of biosynthetic gene clusters through regulatory proteins that respond to such stimuli (Brakhage 2013).

Biological challenges in the form of co-cultivation with other microorganisms may activate silent biosynthetic pathways leading to increased metabolite and mycotoxin production as well as changes in growth rate (Müller et al. 2012; Netzker et al. 2015). For instance, *F. demicellulare* show enhanced production of fusaristatin A which inhibit the growth of its competitor (Li et al. 2016). *F. tricinctum* grown together with *B. substilis* enhance the formation of enniatins and fusaristatin A drastically, and induce the formation of three novel compounds; macrocarpon C, 2-(carboxymehylamino)benzoic acid and (–)-citreoisocoumarinol (Ola et al. 2013). This demonstrates the utility of this approach and confirms the role of SMs as competitive agents.

One explanation as to why BGCs appear silent has been connected to the observation of most *Fusarium* biosynthetic genes reside in non-conserved chromosomal regions associated with low transcriptional activity (Zhao et al. 2014). For instance, in *Fusarium graminearum*, only one out of 14 *PKS* genes and two out of 17 *NRPS* genes are found in conserved regions of the genome, while the remaining genes are located in non-conserved regions. These regions are associated with condensed heterochromatin under regulation of several layers of histone modifications such as methylation and sumoylation (Reyes-Dominguez et al. 2012; Connolly et al. 2013). Loss of histone modifying proteins in *Fusarium* has led to change in expression levels of biosynthetic genes (Connolly et al. 2013; Studt et al. 2017; Kong et al. 2018). Disruption of the heterochromatin methyltransferase KMT6 led to transcriptional activation of four novel putative BGCs in *F. fujikuroi*, and isolation of a novel sesquiterpene (Studt et al. 2016b).

One obvious strategy is to introduce fungi to several different growth conditions and media recipes. To maximize the chance of observing fungal metabolites, a popular strategy is to use different cultivation parameters. This methodology is widely adapted and is often referred to as the One Strain Many Compounds (OSMAC) philosophy (Bode et al. 2002; Nielsen et al. 2011; Hemphill et al. 2017a). However, activation of cryptic biosynthesis pathways is never guaranteed even with a wide variation in substrate compositions and culture conditions (Gaffoor et al. 2005). Properties like pH and nitrogen source are important parameters to control for some metabolite pathways (Linnemannstöns et al. 2002; Kim et al. 2005b). Substituting the nitrogen source glutamine with sodium nitrate in ICI medium lead to the formation of fuarubins instead of bikaverin pigmentation in *F. fujikuroi* (Studt et al. 2012), which emphasizes the importance of standardized growing medium recipes to strengthen reproducibility (Wiemann et al. 2009; Sørensen and Sondergaard 2014). The OSMAC framework has enabled activation of many silent BGCs and led to the discovery of novel compounds. Although useful, discovery of novel compounds can be problematic because testing several culture conditions does not ensure formation of every possible product (Hemphill et al. 2017b; Romano et al. 2018).

1.5 Transformation and gene targeting

Identification of the remaining biosynthetic pathways in *Fusarium* requires an approach utilizing both molecular genetics and analytical chemistry. The majority of studies mentioned in **Table 1** have utilized genetic manipulation to create a link between genes and the formation of a specific biosynthetic metabolite. A prerequisite for use of this approach in *Fusarium* metabolomics was to develop the transformation protocols and tools. Protoplast-mediated transformation (PMT) is the most commonly used transformation system in filamentous fungi. Protoplasts are easy to make and require no special equipment. Freshly germinated hyphae are treated with commercially available enzymes to remove complex cell-wall components in order to release protoplasts (**Figure 5**) (Rodriguez-Iglesias and Schmoll 2015). The protoplasts are usually suspended in an osmotic stabilizing solution containing $CaCl_2$ (**Figure 6AB**). Calcium ions are added to open channels in the cytomembrane and thus promote uptake of free nucleotides (Olmedo-Monfil et al. 2004). Polyethylene glycol (PEG) forms an artificial cell wall and promotes fusion between exogenous nucleotides and protoplasts (**Figure 6C**) (Becker and Lundblad 2001). Transformed protoplasts often require regeneration in osmotic stabilized medium before they are selected. PMT has been applied to transform several *Fusarium* species with high levels of success (**Table 2**). Protoplasts can be mixed with both circular plasmid or linearized DNA. Inconveniently, PMT is known to frequently result in multiple integration events (Proctor et al. 1999; Mullins et al. 2001; Meyer 2008) and has been known to show lower homologous recombination efficiency than other methods (Grallert et al. 1993).

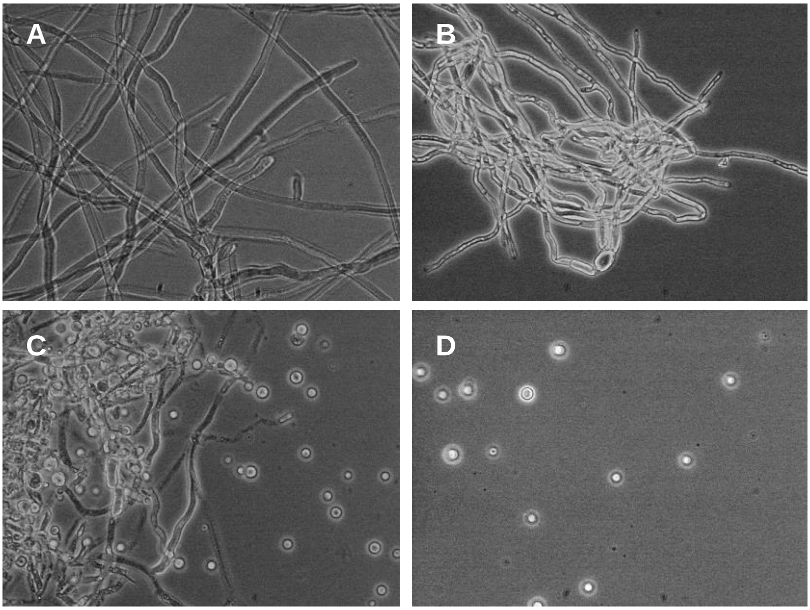

Figure 5 Protoplastation of *F. graminearum* mycelium observed under microscopy. **A.** Freshly germinated hyphae. **B.** 5 minutes after addition of cell wall-degrading enzymes. **C.** Protoplasts are released after 60-90 minutes. **D.** After 120 minutes the protoplasts are harvested and filtered to remove tissue debris.

The gram-negative bacterium *Agrobacterium tumefaciens* is well known for its ability to infect plants and during this process capable of transfer the T-DNA (transfer-DNA) region of the Ti plamid to the genome of the colonized host. The T-DNA regions is bordered by two imperfect inverted repeats (Left and Right Border; LB and RB), and it is possible to introduce exogenous DNA by inserting it between the two border sites in a binary vector (Citovsky et al. 2007). *A. tumefaciens* is also capable of infecting filamentous fungi when *Agrobacterium* virulence genes are induced by added acetosyringone (**Figure 6D**) (Idnurm et al. 2017), and a vast arsenal of binary vectors has been developed for this purpose (Frandsen 2011; Sørensen et al. 2014b). The T-DNA is usually integrated in the fungal genome as a single copy by homologous recombination (Michielse et al. 2005), and has proven to be more stable and efficient than protoplasting (de Groot et al. 1998; Fernández-Martín et al. 2000; Malz et al. 2005). The major bottlenecks in this technique includes the preparation of binary vectors and testing of various technical parameters, as an optimized protocol has to be developed for every species (de Groot et al. 1998; Utermark and Karlovsky 2008; Sørensen et al. 2014b). As for PMT, several *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocols have been developed for several representatives of *Fusarium* (**Table 2**).

Table 2 Representative protoplast-mediated transformation (PMT) and *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocols. Adapted from **Paper 1** (Nielsen et al. 2019c).

Species /strain	PMT	ATMT
F. solani f.sp *pisi*	(Soliday et al. 1989)	(Romans-Fuertes et al. 2016)
F. solani f.sp *phaseoli*	(Marek et al. 1989)	
F. solani f.sp *curcurbitae*	(Crowhurst et al. 1992)	
F. graminearum PH-1	(Connolly et al. 2018)	(Frandsen et al. 2012)
F. graminearum A3/5	(Wiebe et al. 1997)	
F. fujikuroi	(Linnemannstöns et al. 2002)	
F. semitectum	(Jin et al. 2010)	(Jin et al. 2010)
F. venenatum	(Song et al. 2004)	(de Groot et al. 1998)
F. pseudograminearum	(Gardiner et al. 2012)	(Tobiasen et al. 2007)
F. heterosporum	(Kakule et al. 2013)	
F. verticilloides	(Brown et al. 2012)	
F. pallidoroseum	(Naseema et al. 2008)	
F. pulocaris	(Salch and Beremand 1993)	
F. culmorum		(Tobiasen et al. 2007)
F. oxysporum f. sp. *lycopersici*		(Takken et al. 2004)
F. oxysporum O-685		(Mullins et al. 2001)
F. curcinatum		(Covert et al. 2001)
F. avenaceum		(Sørensen et al. 2014b)

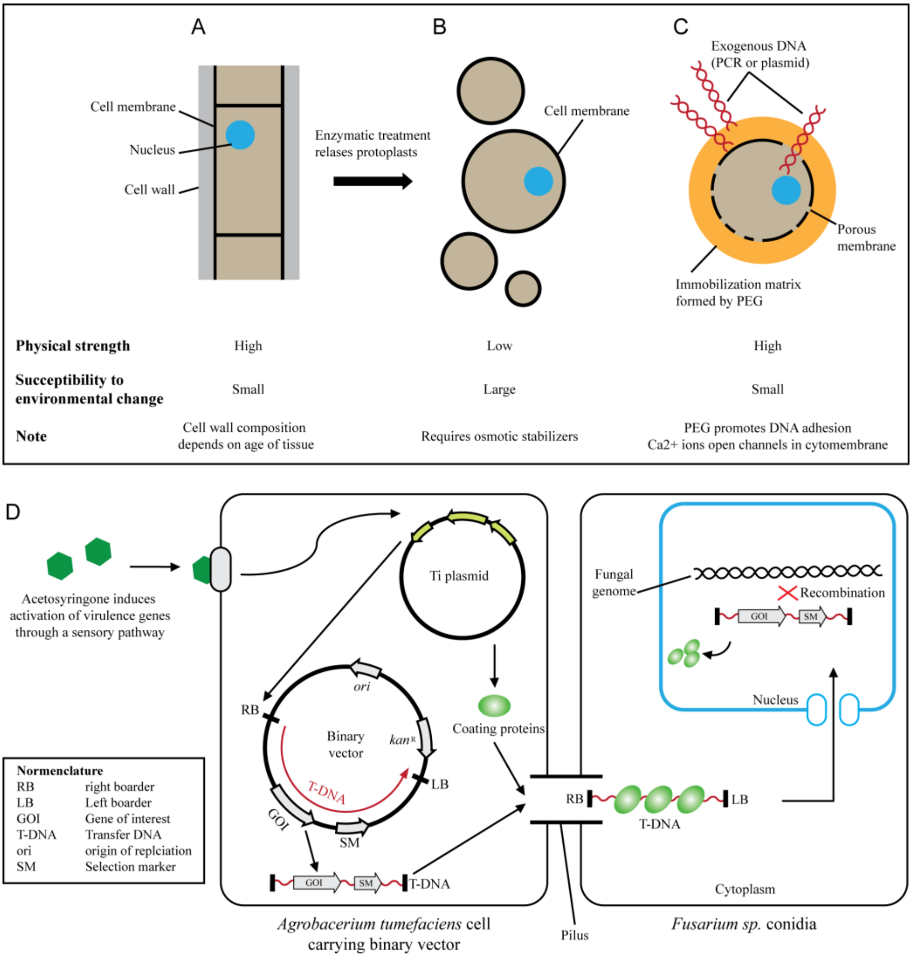

Figure 6 Overview of Protoplast-Mediated Transformation and *Agrobacterium tumefaciens*–Mediated Transformation of filamentous fungi. **A.** Mycelial tissue comprising a thick cell wall. **B.** Enzymatic treatment of mycelium releases protoplasts encapsulated by cytomembrane and no cell wall. **C.** Protoplast transformation. Polyethylene glycol (PEG) can form a molecular bridge between cell and exogenous nucleotides. DNA uptake is possible through a porous membrane. **D.** Overview of T-DNA delivery to the nucleus of *Fusarium* conidia. Copied from **Paper 1** (Nielsen et al. 2019c)**.**

1.5.1 Homologous recombination enables gene targeting

Recombinant DNA can integrate into the genome either randomly, guided by non-homologous end-joining repair or targeted catalyzed by homologous recombination repair (Symington 2002). In Aspergilli, non-homologous end-joining deficient mutants were developed to favor homologous recombination thus enabling high efficiency gene targeting (Nayak et al. 2006). One benefit to working with *Fusarium* is the naturally high affinity for homologous recombination frequency enabling gene targeting without the need for down regulating non-homologous end-joining enzymes (Frandsen et al. 2012; Connolly et al. 2018). In order to utilize gene targeting guided by homologous recombination repair vectors should be equipped with one or two segments of nucleotide sequence identical to the target locus. The amount of homologous nucleotides required for homologous recombination varies from species to species. The exact optimal size of homologous segments has rarely been tested, although one study found a minimum of approximately 800 bp lead to 93 % homologous recombination events (Maier et al. 2005). Later, other studies have adapted vectors containing 800-1500 bp gene targeting segments (Oide et al. 2014; Wollenberg et al. 2017; Bahadoor et al. 2018). However, heterologous or ectopic recombination is a common phenomenon (Frandsen et al. 2012; Sørensen et al. 2014b), resulting in unchanged product formation levels and phenotype. Validation of transgenic fungi with diagnostic PCR, Southern blot or sequencing is necessary in order to verify intended targeted integration in mutants (Proctor et al. 1999; Malz et al. 2005). Transformation vectors typically contain an antibiotic selection marker (e.g. *hygB* or *nptII*) under regulation of an inducible or constitutive fungal promoter sequence (**Figure 7**) (e.g. *A. nidulans trpC* or *gdpA* promoter). Alternatively auxotrophic selection markers have been developed for *F. graminearum* (Connolly et al. 2018).

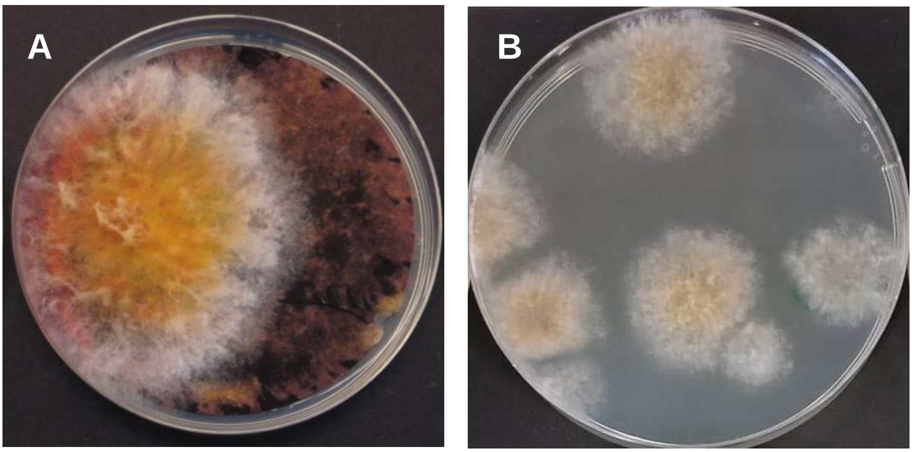

Figure 7 Transformation of *F. graminearum* with resistance marker-containing cassettes. **A.** *Agrobacterium tumefaciens*-mediated transformation plate. The transformation reaction containing both vector carrying *Agrobacterium* and fungal macroconidia are spread onto black filter paper. The filter is transferred onto new media for consecutive selection rounds; first killing off the *Agrobacterium* donor strain, and later, untransformed fungal conidia. **B.** Resistant colonies emerge following transformed protoplasts are overlaid with selective agarose medium.

1.6 Low hanging fruits: linking metabolites produced in laboratory medium

A significant portion of fungal SMs can be produced in laboratory cultures. To identify biosynthetic genes, a simple but effective strategy has been to identify putative gene candidates, deleting or disrupting the genes, and then determine the SM complement. Absence of a specific metabolite in a mutant provides evidence that this gene is crucial for the respective biosynthetic pathway. This concept termed *gene replacement*, sometimes referred to as *knock-out* (KO), is based on genomic insertion of a vector element containing homology to the targeted gene through homologous recombination. To create a gene-replacement cassette PCR reactions are performed on the recipient species genomic DNA as template. The primers should be designed to amplify homologues targeting segments inside the gene (gene disruption) or on either side of the gene (gene replacement). This process yields a mutant either carrying a truncated version or lacking the biosynthetic gene entire which can be compared to the progenitor strain (Proctor et al. 1999; Malz et al. 2005). A successful knock-out mutant is thus unable to express the biosynthetic gene, and investigation of phenotype changes can be initiated. Suitable targets for gene disruption in SM studies are core biosynthesis genes. Disruption vectors containing a single segment homologous to the target gene are rapidly prepared and introduced to fungal genome by a single cross-over recombination event guided by either PMT (Gaffoor et al. 2005; Gaffoor and Trail 2006; Brown et al. 2012) or ATMT (Malz et al. 2005) (**Figure 8A**).

1.6.1 Targeted gene replacement

A more popular disruption strategy is based on vectors containing two homologous segments to the target gene separated by a selection marker gene (**Figure 8B**). This enables replacing a large portion or the entire biosynthetic gene with the selection marker. Gene replacement has been carried out in most *Fusarium* spp. guided by either PMT of *F. fujikuroi* and *F. venenatum* (Proctor et al. 1999; Song et al. 2004; Wiemann et al. 2009; Niehaus et al. 2014a; Janevska et al. 2016; Studt et al. 2016a) or ATMT of *F. graminearum*, *F. pseudograminearum*, *F. solani*, *F. avenaceum*, and *F. semitectum* (Frandsen et al. 2006, 2016; Tobiasen et al. 2007; Ma et al. 2010; Sørensen et al. 2014b, a; Romans-Fuertes et al. 2016; Wollenberg et al. 2017; Bahadoor et al. 2018). Recombination between vector carrying two homology segments and the genome can resolve in four possible outcomes. The four recombination possibilities between genome and a vector are; double cross-over leading to gene replacement, integration of the plasmid in the 5' end of the gene by a single cross-over, integration in the 3' end of the gene by a single cross-over event, or plasmid integration in both ends of the gene. Indeed, mixed recombination events were observed in *F. fujikuroi*. In one study, out of 16 mutants only one displayed correct gene replacement through double recombination, while 15 mutants had experienced integration of the entire vector in one or more copies, disrupting *PKS24* (Proctor et al. 1999).

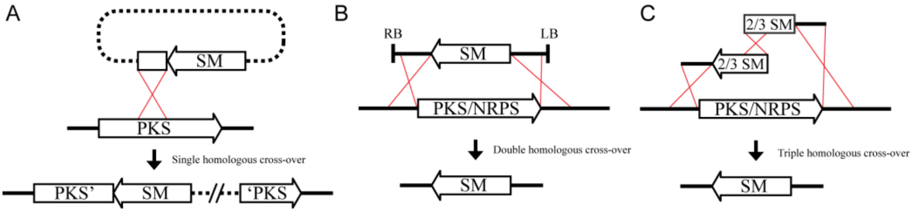

Figure 8 Targeting disruption and gene replacement strategies. **A.** Gene disruption by integration of a plasmid containing segment homologous to part of biosynthetic gene. **B.** Gene replacement by two homologous recombination events (double cross-over) replacing the entire open reading frame with the cassette. **C.** Split marker gene replacement with two nucleotide fragments. Figure nomenclature: PKS = polyketide synthase, NRPS; non-ribosomal peptide synthetase, SM; selective marker gene. Copied from **Paper 1** (Nielsen et al. 2019c).

ATMT with *A. tumefaciens* strain LBA4404 was used for gene replacement experiments in *Fusarium* spp. with great success (Idnurm et al. 2017). Based on the ATMT protocol developed by Sacha Malz and collegues (Malz et al. 2005), a vector system for targeted gene deletion was developed for *F. graminearum* (Frandsen et al. 2006, 2008, 2012), allowing characterization of the aurofusarin (*PKS12*) and fusarubins (*PKS3*) pigment biosynthesis, and many more (Sørensen et al. 2014a; Wollenberg et al. 2017).

Lastly, gene replacement has been performed by transforming *Fusarium* protoplasts with PCR products comprising a selection cassette and two segments homologous to target gene. Catlett *et al*. (2003) introduced the split marker system where two PCR products each comprised a 3' or 5' homologous target region with each two-thirds of the selection marker, together capable of forming an intact deletion cassette when combined through homologous recombination (**Figure 8C**) (Catlett et al. 2003). The split marker-based transformation approach is believed to increase the frequency of homologous integration and decrease the risk of ectopic and tandem integration events in fungi (Catlett et al. 2003; Chung and Lee 2015).

Gene replacement is a powerful tool to link genes to function and entire pathways can be resolved in this way. Not only the core synthase can be identified but the contribution of the other genes in the same cluster to the final product can be determined (Frandsen et al. 2006, 2016; Wiemann et al. 2009; Studt et al. 2012, 2016a; Kakule et al. 2013). But it is important to bear in mind that for a successful outcome of this strategy the fungus must produce the target compound under the cultivation conditions used. Thus, gene disruption and knock-outs cannot be applied to silent gene clusters, or biosynthetic pathways yielding small amounts of product.

1.7 Targeted activation

With the introduction of sequencing, identification of gene clusters have become trivial – however discovering the compounds produced by the majority of these clusters remains challenging, especially when the pathways are silent when cultivated in the laboratory. In parallel to targeted gene replacement targeted gene activation is used to discover new biosynthetic pathways in *Fusarium* spp.. Core synthase genes like *PKS* and *NRPS* genes make ideal targets for targeted gene activation. A vector is prepared containing a constitutive promoter and a selection marker between two segments for targeted integration upstream of the biosynthetic gene in question (**Figure 9A**). USER cloning has been demonstrated to enable quick assembly of such vectors for targeted promoter replacement in *F. graminearum* (Frandsen et al. 2008). The pRF-HU2E vector can be easily equipped with suitable homologous sequences upstream from the target gene, enabling promoter swapping to the constitutive *A. nidulans* PgdpA in front of PKSs and NRPSs. This activated production of gibepyrones A, B, D, and G and polypyrone B (*PKS8*) (Westphal et al. 2018a), chrysogine (*NRPS14*) (Wollenberg et al. 2017), orsellinic acid and orcinol (*PKS14*) (Jørgensen et al. 2014), and three novel bostrycoidin anthrones (*PKS3*) (Frandsen et al. 2016) not detected in wild type *F. graminearum*. Over-expression of *NRPS4* lead to an increase in surface hydrophobicity, but no specific SM responsible for this phenotype could be identified by chemical analyses (Hansen et al. 2012a). Comparison of knock-out mutants to the wild type in the *F. heterosporum PKS69* pathway failed to identify differences in the SM profile on different growing media. But fusing a copy of the *fsdS* (*PKS69*) gene with the constitutive equisetin synthase (*PKS18*) promoter in a mutant construct resulted in formation of fusaridione A, which is likely the first intermediate in the biosynthetic pathway (Kakule et al. 2013).

Targeted activation can also aim to activate transcriptional regulator genes. Biosynthesis gene clusters often contain a Zn(II)2Cys6-domain gene that act as a cluster specific transcription factor (TF) protein (Brown et al. 2007; Brakhage 2013). Examples are the Gip2, Bik5 and Fsr6 proteins controlling pigment biosynthesis in *F. graminearum* and *F. fujikuroi* (Kim et al. 2006; Studt et al. 2012; Wiemann et al. 2013). Exchanging the native promoter of putative transcription factor *APS2* for the β-tubulin promoter in *F. semitectum* resulted in up-regulation of the *NRPS31* cluster genes and increased formation of apicidin F (Jin et al. 2010). Over-expression of a BGC-associated TF in *F. solani* led to the discovery that the first polyketide intermediate in perithecial pigmentation synthesis is prephenalenone (**Paper 4**) (Nielsen et al. 2019b). Analysis of BGC promoter regions with the Regulatory Sequence Analysis Tool (RSAT) can reveal conserved TF binding motifs suggesting that expression is regulated by a single Zn(II)2Cys6 binuclear transcription factor (van Helden et al. 2000; Sørensen et al. 2012a; Sieber et al. 2014; Frandsen et al. 2016).

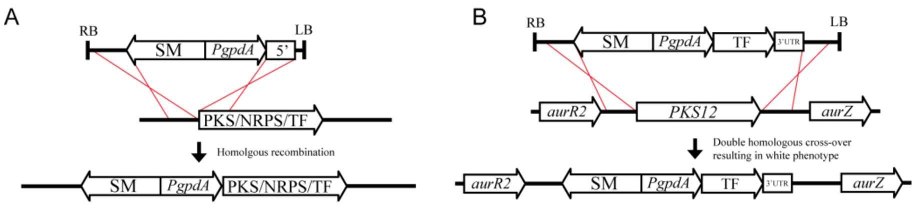

Figure 9 Targeted activation strategies with vectors containing two flanking homology segments **A.** in locus promoter replacement upstream of a biosynthetic gene. **B.** A copy of a transcription factor gene (TF) is fused to the *PgpdA* promoter and inserted at an ectopic genomic position, here exemplified with the *PKS12* locus, yielding an albino mutant phenotype. Figure nomenclature: PKS = polyketide synthase, NRPS; non-ribosomal peptide synthetase, SM; selective marker gene, TF; transcription factor gene, 3'-UTR; 3'-untranslated region. Copied from **Paper 1** (Nielsen et al. 2019c).

To ease the process of identifying overexpression mutants, one method has been to amplify the transcription factor genes including the native terminator and fusing it to the pRF-HUEA expression cassette with homologous targeting segments in the *F. graminearum PKS12* locus, resulting in albino mutants (**Figure 9B**) (Frandsen et al. 2008, 2016). This system was used to overexpress the putative expression factor *Fsr7*, resulting in increased formation of three novel toxins; fusarielins F, G and H (Sørensen et al. 2012a). To ensure high expression, targeted integration into a non-coding locus adjacent to the β-tubulin gene in *F. graminearum* (Josefsen et al. 2012) has been used for *aurR1* overexpression, enabling overproduction of aurofusarin biosynthesis metabolites including novel putative shunt products (Westphal et al. 2018b). Combined overexpression of *PKS39* and the cluster specific transcription factor *APS2* gave a 10-fold increase in metabolite production and enabled characterization of a novel group of metabolites; fujikurins B, C and D (Wiemann et al. 2013; Von Bargen et al. 2015).

Targeted gene activation can be directed towards proteins controlling secondary metabolism on a higher level of regulation (Tudzinsky & Janevska 2018). In *Asperillus* spp., overexpression of the global regulator protein LaeA lead to increased levels of both polyketide and non-ribosomal peptide SMs (Bok and Keller 2004). Likewise did deletion of the *F. verticilloides leaA* orthologue *LAE1* lead to decreased SM formation levels (Butchko et al. 2012). Such strategies are effective when used in tandem with other methods to promote formation of SMs (Butchko et al. 2012; Giese et al. 2013), and can contribute to the activation of silent BGCs.

1.8 Heterologous expression

Activation in natural host has been demonstrated to be a viable strategy for filamentous fungi with developed and available transformation systems. Many species of filamentous fungi cannot grow in the laboratory or are difficult to transform (Chávez et al. 2015), and cannot benefit from molecular genetics approaches. The lack of available tools for genetic modification of most species thus hinders pathway discovery. Heterologous expression of pathway genes in a model hosts or cell factories enables product discovery, pathway elucidation and production and isolation (Lazarus et al. 2014; Anyaogu and Mortensen 2015). Heterologous expression serves two additional purposes; the expression host gives access to a large genetic manipulation toolbox, and; any produced metabolites are easily distinguishable against background metabolism. Heterologous expression does not rely on the donor strain being transformable and is perhaps therefore considered the most universal solution to produce natural products from silent BGCs and uncultivable microorganisms (Ongley et al. 2013; Chiang et al. 2013).

Heterologous expression of fungal biosynthetic genes has been applied successfully in several studies utilizing the filamentous fungi *Aspergillus oryzae* or *A. nidulans* as expression hosts (**Table 3).** The workflow can be split into two parts; cloning the biosynthetic genes into a vector system, and; introducing the vector into the filamentous fungi host (Anyaogu and Mortensen 2015; Alberti et al. 2017).

The biosynthetic clusters can be assembled into cosmids which keeps the gene clusters intact (Sakai et al. 2012). However, isolation of a cosmid clone containing all desired biosynthetic genes is never guaranteed (Sakai et al. 2008). Alternatively can PCR-amplified biosynthetic genes be assembled into vectors through USER cloning (Hansen et al. 2011; Nielsen et al. 2013), Restriction/ligation (Heneghan et al. 2010; Fujii et al. 2011), yeast recombination (Yin et al. 2013), all which enables fusing genes to strong promoters to ensure expression (Itoh et al. 2012; Chiang et al. 2013). All genes necessary for product biosynthesis should be included in the workflow (Sakai et al. 2012). This often creates a bottleneck as some BGCs contain 10 or more genes, because cloning and transforming each gene results in several transformation iterations (Sakai et al. 2012; Fujii et al. 2016). Ingenious cloning systems have been developed to ensure transfer of several genes per transformation reaction (Nielsen et al. 2013; Chiang et al. 2013; Fujii et al. 2016). However, cloning and introduction of all necessary cluster genes is still difficult and the studies reported so far have failed to demonstrate reconstruction and heterologous expression of gene clusters larger than 28 Kbp without utilizing cosmid assembly (Sakai et al. 2012; Yin et al. 2013). Cases of heterologous expression in filamentous fungi have so far been restricted to *PKS* and *TC* genes, as *NRPSs* are enormous and heterologous expression of entire NRPS pathways may not be a feasible option. Therefore, there is a great need for further development of the heterologous expression methods in order to handle larger gene clusters and therefore enable scientists to tap into the many silent BGCs of filamentous fungi.

Recombinant biosynthetic genes are often inserted into the genome of the recipient host with gene targeting (**Figure 10**). Targeted genomic integration provides a few benefits, such as enabling control of copy number and mitotic stability of genes (Palmer and Keller 2010; Mikkelsen et al. 2012). However, in the context of natural product discovery ensuring expression is key, and unguided expression vectors have been implemented with success in Aspergilli (Fujii et al. 1996, 2011, 2016; Sakai et al. 2008; Heneghan et al. 2010; Itoh et al. 2010, 2012).

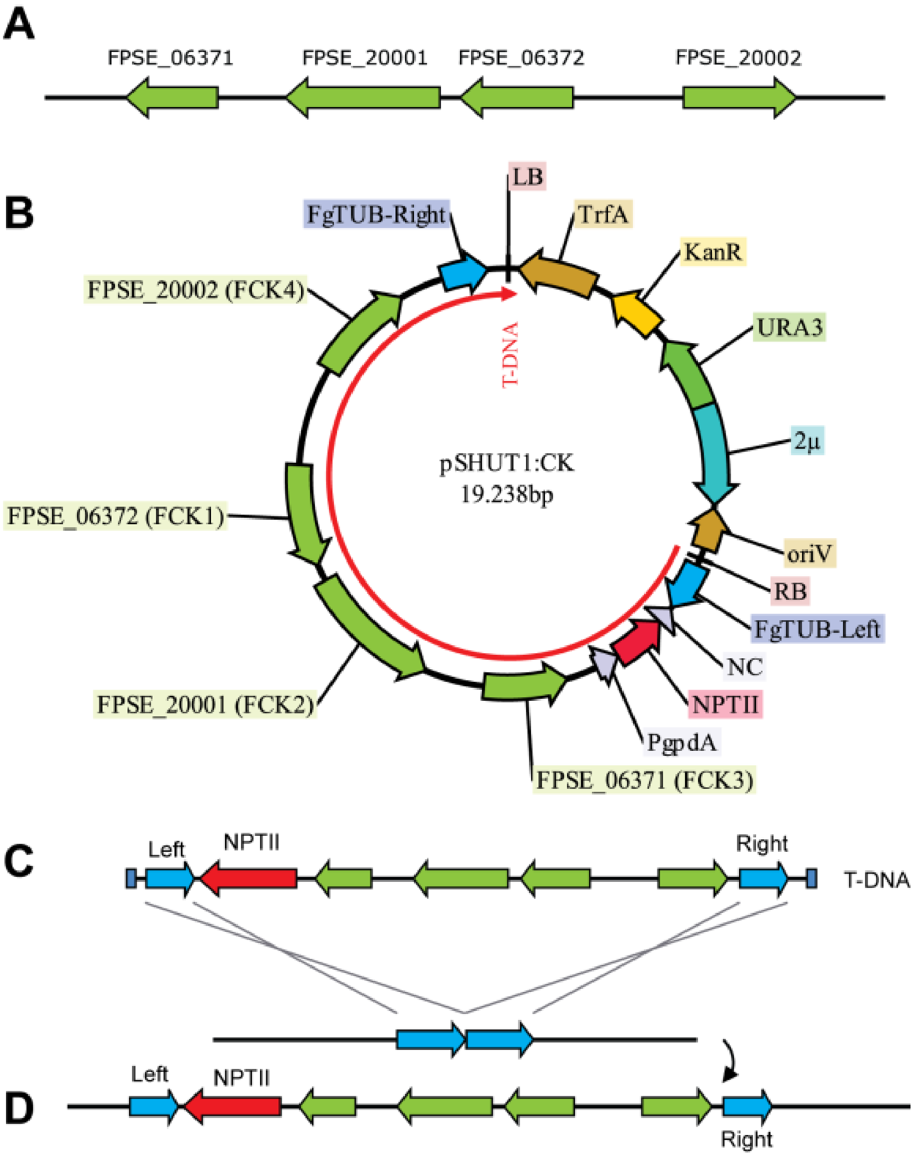

Figure 10 Workflow for introducing foreign biosynthetic genes in a filamentous fungal expression host. **A.** The *F. pseudograminearum FCK* gene cluster comprises four genes. **B.** The plasmid pSHUT1:CK comprise elements for *Agrobacterium tumefaciens*-mediated transformation of the inserted *FCK* cluster. The transfer-DNA segment is marked in red. **C.** Transformed filamentous fungal conidium receives and introduces a copy of the Transfer-DNA cassette by homologous recombination between flanking targeting nucleotide segments and the target genome position. **D.** Resolving fungal genome following double cross-over recombination. Adapted from **Side project 2** (Sørensen et al. 2018).

Table 3 Cases of heterologous expression of biosynthetic gene clusters in filamentous fungi. HR; yeast homologous recombination cloning.

SM pathway	Type	Genes	Host	Integration	Transf. iterations	Reconstitution	Insertion size	Vector cloning	Origin Sp.	Study
Geodin	PKS	13	*A. nidulans*	Targeted	2	*PgpdA::gedR* (TF)	25000	USER	*A. terrus*	(Nielsen et al. 2013)
Asperfuanone	PKS	6	*A. nidulans*	Targeted	6	alcA:: 6 pathway genes	24965	USER	*A. terrus*	(Chang et al. 2013)
Neosartoricin B	PKS	12	*A. nidulans*	Targeted	2	PgpdA::TESG_06706 (TF)	28000	SOE-PCR, HR	*T. tonsurans*	(Yin et al. 2013)
Mycophenolic acid	PKS	1	*A. nidulans*	Targeted	1	PgpdA::*mpaC* (PKS)	7745	USER	*P. brevicompactum*	(Hansen et al. 2011)
Monacolin K	PKS	9	*A. oryzae*	Vector	3	pgk::*laeA*	45000	cosmid	*M. pilosus*	(Sakai et al. 2012)
Terrequionone A	Amino acid-isoprenoid	5	*A. oryzae*	Vector	2	pgk::*laeA*	12000	fosmid	*A. nidulans*	(Sakai et al. 2008)
Citrinin	PKS	6	*A. oryzae*	Vector /random	2	trpC::*ctnA* (PKS)	20000	cosmid	*M. purpureus*	
Aphidicolin	Terpene	4	*A. oryzae*	4 vectors	1 to 3	PamyB:: four pathway genes	7100	R+L	*P. betae*	(Fujii et al. 2011)
Tenellin	PKS-NRPS	4	*A. oryzae*	3-4 vectors	1	PamyB:: four pathway genes	17449	R+L	*B. bassiana*	(Heneghan et al. 2010)
Pyripyropene	Meroter-penoid	2	*A. oryzae*	2 vectors	2	PamyB:: *all genes*	<9300	Gateway, R+L	*A. fumigatus*	(Itoh et al. 2010, 2012)
6-MSA	PKS	1	*A. nidulans*	vector	1	PamyB::atX (PKS)	7588	R+L	*A. terrus*	(Fujii et al. 1996)
Solanapyrone A	PKS	4	*A. oryzae*	vector	4	PamyB:: *all genes*	<9608	R+L	*A. solani*	(Fujii et al. 2016)
Ferrirhodin	NRPS	1	*A. oryzae*	Random integration	1	PamyB::*FSN1* (NRPS)	14121	HR	*F. sacchari*	(Munawar et al. 2013)
***Fusarium* cytokinin**	Isoprene	4	*F. graminearum*	Targeted	1	none	9944	HR	*F. pseudograminearum*	(Nielsen et al. 2019d) ***Paper 3***
W493 B	NRPS + PKS	7	*F. graminearum*	Targeted	1	none	54471	HR	*F. pseudograminearum*	
(Prephenalenone)	PKS	11	*F. graminearum*	Targeted	1	none	26076	HR	*F. solani*	(Nielsen et al. 2019b) ***Paper 4***

1.8.1 Heterologous expression in yeast

Another feasible expression host is the unicellular yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* (Gao et al. 2013; Cochrane et al. 2016). *S. cerevisiae* is easily manipulated and has a broad fan of available expression plasmids as well as efficient gene targeting through homologous recombination (Lazarus et al. 2014; Alberti et al. 2017). In contrast to filamentous fungi do yeasts contain low metabolic background (Siddiqui et al. 2012). This enables easy metabolite detection and minimizes undesirable side-reactions between foreign genes and endogenous metabolome. However, this also means yeast is not naturally geared for SM formation and sometimes contain insufficient concentrations of precursors (Mutka et al. 2006), which is also why previous endeavors usually include some optimization of precursor production (Ishiuchi et al. 2012; van Rossum et al. 2016). However, the genes of yeast contain naturally few introns (Spingola et al. 1999) and have intron splicing recognition different from that of filamentous fungi (Kupfer et al. 2004). A prerequisite to yeast expression is therefore complete removal of introns (Tsunematsu et al. 2013). In one case were genes from *F. graminearum PKS12* biosynthetic pathway introduced and heterologously expression in *S. cerevisiae*. Three synthesized or assembled intron-less recombinant genes including the *PKS12* together with the A. *fumigatus* 4'-phophopentetheinyl transferase *npgA* were assembled into yeast expression vectors. The yeast strains were capable of producing the first intermediate compounds in the aurofusarin pigmentation pathway (Rugbjerg et al. 2013).

One major advantage to working with yeast is the efficient plasmid construction by *in vivo* DNA recombination (**Figure 11**) (Ma et al. 1987). Yeast homologous recombination promotes error-free repair of double stranded nucleotide molecules (Krogh and Symington 2004), which can be exploited to assemble recombinant DNA fragments comprising small stretches of identical nucleotide sequences (Kuijpers et al. 2013). Furthermore, this has enabled reliable assembly and cloning of chromosomal segments up to hundreds of thousands of base pairs (Noskov et al. 2011; Kouprina and Larionov 2016). A recent and ambitious study reported rapid reconstruction of 41 fungal biosynthetic pathways, although detection of novel metabolites was found in only 22 of those mutants. Harvey *et al* (2018) blame intron recognition and accurate removal of these being the major bottleneck in obtaining gene functionality in yeast (Harvey et al. 2018).

Heterologous expression of *Fusarium* genes in yeasts provides a rapid method for screening or producing biosynthetic products. Likewise, has heterologous expression in *E. coli* provided a feasible method for identifying the novel *F. fujikuroi* sesquiterpene (–)-germacrene D (Niehaus et al. 2017b). Heterologous expression in bacteria however may not be feasible for other fungal SMs when the synthesis require specialized compartmentalization (Roze et al. 2011).

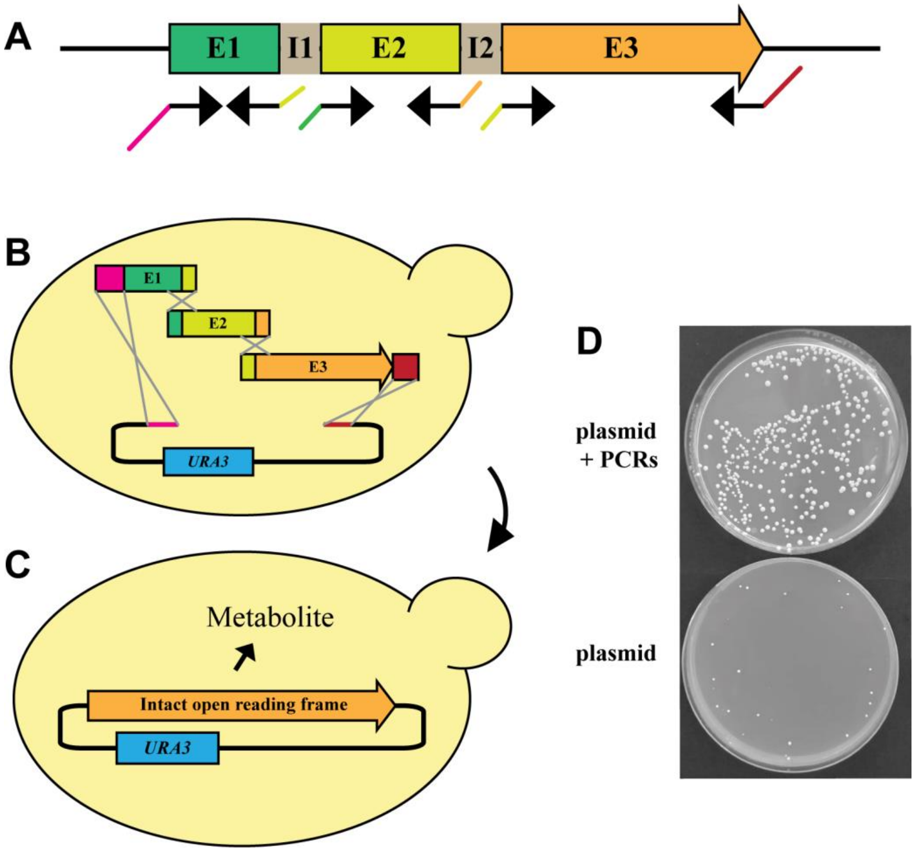

Figure 11 An intron-less version of a filamentous fungal synthase is assembled through yeast homologous recombination. **A.** The synthase gene comprises two introns (I1 and I2) and three exons (E1–3). **B**. PCR-amplification of three exons performed with primers with tails containing homology to either plasmid multiple-cloning site or neighboring exon. **C.** Identical sequences longer than 15 bp are recognized by the yeast DNA-repair mechanisms which guides assembly of three fragments and the linearized plasmid in a transformation associated recombination reaction. **D.** A recombined and circular plasmid molecule will be able to propagate and rescue uracil auxothrophy in transformants. As a control, we usually transform yeast cells with the linearized vector and omit the PCR inserts. The control experiment produces significantly (10 to 100-fold) fewer colonies as the vector is not capable of repairing itself without the homology-containing inserts.

2. Summary of results and discussions from papers

This section will consist of a summary of the results and discussions from the three original research papers included in this thesis:

- A new vector system for ectopic gene expression in the crop pathogen *Fusarium solani* (**Paper 2**, submitted to Journal of Fungi)

- Heterologous expression of intact biosynthetic gene clusters in *Fusarium graminearum* (**Paper 3**, submitted to Fungal Genetics & Biology)

- The final piece to the polyketide pigmentation puzzle in *Fusarium solani* (**Paper 4**, manuscript in draft)

2.1 A new vector system for ectopic gene expression in the crop pathogen *Fusarium solani*

The genome of crop pathogen *Fusarium solani* f. sp. *pisi* comprises 6 PKSs and 4 NRPSs confined to the *F. solani* species complex (Brown 2016, Hansen 2015), out of which only *NRPS31* is linked to an isolated chemical compound (Graziani et al. 2004; Romans-Fuertes et al. 2016). This creates potential for discovery and characterization of novel biosynthetic products. Describing the molecular biology of this fungus is halted by the lack of available molecular tools. In this study we explain the development and implementation of a vector system enabling ectopic overexpression of genes in *F. solani*. The vector was constructed utilizing yeast recombination cloning, and is equipped with two homology segments for targeted integration in a non-coding locus downstream of the *F. solani* β-Tubulin gene. As a proof of concept, a vector was prepared for expression of the fluorescent reporter gene *eYFP* under control of the constitutive TEF1 promoter (**Figure 12A**). In order to successfully transform *F. solani*, we turned to ATMT with the virulent *A. tumefaciens* AGL-1 strain (**Figure 12B**), which yielded 2.5 colonies per 1 million spores (Romans-Fuertes et al. 2016). The T-DNA cassette had successfully integrated in the genome of isolated mutants and fluorescent microscopy confirmed the *eYFP* phenotype (**Figure 12C**). We expect the presented vector system will serve as a tool for targeted activation strategies and thus contribute to the characterization of natural products from *F. solani*.

2.1.1 Additional results and outlook

In this subsection, additional preliminary results that have been generated during the PhD utilizing the pSHUT4 vector system are presented. These results will be published in a separate publication in the future when structural elucidation of compounds has been completed.

A similar vector system has been used to activate polyketide biosynthesis through overexpression of TFs in *F. graminearum* (Frandsen et al. 2016; Westphal et al. 2018b). In order to explore the biosynthetic potential of *F. solani* we searched the genome for *PKS* gene clusters containing putative cluster-specific transcriptional regulators. Six gene clusters were identified (*PKS3*, *PKS22*, *PKS30*, *PKS31*, *PKS33*), comprising a total of 11 candidate TF genes (**Table 4**). Putative transcription factors were chosen based on; predicted protein function (GO-terms, NCBI conserved domain search), and whether or not the gene is located inside or near the predicted gene cluster frame (CASSIS, ClusterFinder). TF genes were amplified from genomic DNA and assembled into pSHUT4, which was confirmed with sequencing. *F. solani* mutants overexpressing TF genes were constructed according to the workflow presented in **Paper 2** (Nielsen et al. 2019a)**.**

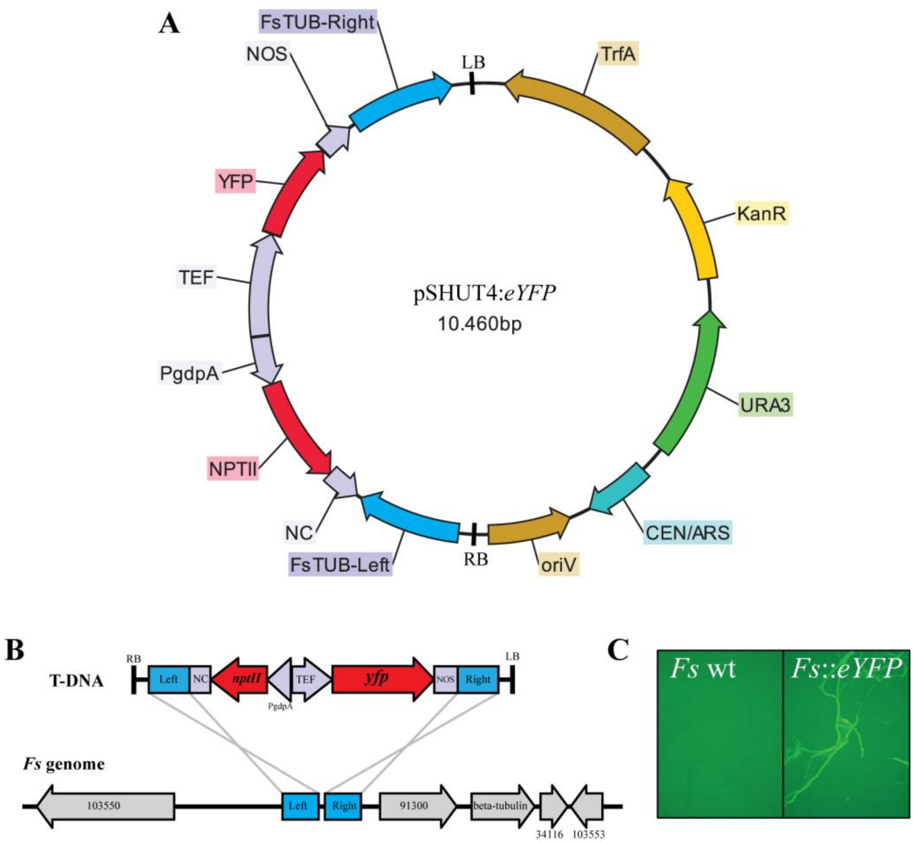

Figure 12 Transformation system utilized for *F. solani*. **A.** Plasmid map of the *Agrobacterium tumefaciens*-mediated transformation vector pSHUT4::*eYFP* **B.** Transfer-DNA cassette integrating in the β-Tubulin locus of *F. solani* via homologous recombination. **C.** Fluorescence microscopy of mycelium samples of wild type *Fusarium solani* and *eYFP* expressing transformant.

A clear change in phenotype was observed in OE::*tf3B* transformants (**Figure 13**), comparable to the black *F. graminearum* OE::*PKS3* mutant phenotype (Frandsen et al. 2016). In future experiments, these mutants will undergo chemical analysis in search of formed polyketide products. Additionally, these mutants might serve as a starting point for biosynthetic pathway elucidation studies.

Table 4 Candidate transcription factors identified in close proximity to polyketide biosynthetic gene clusters (BGC) in the genome of *F. solani*

Cluster	Nickname	Annotation	Size (bp)	Predicted function	Inside BGC?
PKS3	*tf3A*	NECHADRAFT_85473	2.103	Fungal Zn-binding TF	No
	tf3B	*pglR* orthologue (no annotation)	1.167	Fungal Zn(2)-Cy6 binuclear TF	No
PKS22	*tf22*	NECHADRAFT_82314	1.571	Fungal Zn-binding transcription factor	Yes
PKS29	*tf29A*	NECHADRAFT_37376	3.052	DNA-binding response regulator	Yes
	tf29B	NECHADRAFT_78504	1.221	Fungal Zn(2)-Cy6 binuclear TF	No
	tf29C	NECHADRAFT_78523	2.429	Fungal Zn-binding TF	No
PKS30	*tf30*	NECHADRAFT_78422	2.578	Zinc-binding, DNA-binding protein	Yes
PKS31	*tf31A*	NECHADRAFT_53038	2.040	Fungal Zn(2)-Cy6 binuclear TF	Yes
	tf31B	NECHADRAFT_87205	1.848	Transcription factor activity protein	No
PKS33	*tf33A*	NECHADRAFT_94027	1.377	Fungal Zn(2)-Cy6 binuclear transcription factor	Yes
	tf33B	NECHADRAFT_81059	2.561	Fungal Zn(2)-Cy6 binuclear transcription factor	Yes

The vector system was successfully applied for targeted activation and over production of *F. solani* polyketides. In future studies it might be feasible to utilize this strategy to facilitate activation of cryptic biosynthetic pathways *e.g.* TFs found in the *NRPS* gene clusters of *F. solani*.

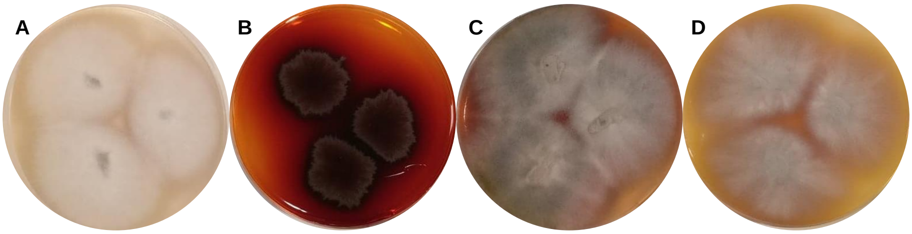

Figure 13 Distinct phenotypes observed amongst *F. solani* overexpression (OE) transformants (**Table 4**) grown on yeast extract-peptone-dextrose medium. **A.** *F. solani* progenitor strain 77-13-4. **B.** OE::*tf3B*. **C.** OE::*tf29A*. **D.** OE::*tf33B*. Other mutants displayed no significant change in phenotype in comparison to the progenitor strain.

2.2 Heterologous expression of intact biosynthetic gene clusters in *Fusarium graminearum*

Heterologous expression of a natural product BGC in a filamentous fungi host is divided into two tasks: cloning the gene cluster into a plasmid vector, and transforming the gene cluster into the genome of the host. In this study, we chose to work with yeast recombination based cloning as this technique has proven feasible for correct assembly of large plasmids from PCR fragments through *in vivo* homologous recombination (Kuijpers et al. 2013). The chromosomal position plays a role in the activation of BGCs in filamentous fungi (Connolly et al. 2013). Furthermore, it is generally considered beneficial to control the integration position of foreign genes, as random integration may results in unpredictable pleiotropic effects that complicate the following analysis (Palmer and Keller 2010; Anyaogu and Mortensen 2015). For this purpose, we selected the well-described strain *F. graminearum* which has proven to enable high gene targeting efficiency through homologous recombination (Frandsen et al. 2012). In this study we focused our efforts towards preparing a vector system which could be applied in both yeast recombination assembly of large fragments, and targeted insertion in the *F. graminearum* genome. The targeted integration locus chosen was a non-coding region upstream the β-Tubulin gene (Josefsen et al. 2012; Westphal et al. 2018b). The 54.4 Kbp *F. pseudograminearum W493* (PKS40-NRPS32) cluster (Sørensen et al. 2014a) was chosen as a test case for proof of concept (**Figure 14AB**). Following protoplast PMT, we isolated two W493 B-producing *F. graminearum* mutants (**Figure 14CD**). To our knowledge, this is the first case of reconstruction and heterologous expression of an intact, multigene, non-ribosomal lipopeptide gene cluster in filamentous fungi. The study separates itself from previously reported cases in more than one way. Heterologous expression studies have demonstrated successful cloning and heterologous expression of terpene and polyketide products from multigene pathways in *A. nidulans*, *A. oryzae* (**Introduction, Table 3**), or *S. cerevisiae*. Previously, several transformation iterations had to be carried out in order to ensure introduction of all essential pathway genes (Yin et al. 2013; Chiang et al. 2013). Alternatively have individual genes been assembled into separate expression vectors and co-transformed into the fungus (Heneghan et al. 2010; Fujii et al. 2016). Due to the size limitations of plasmids and PCR reactions (Nielsen et al. 2013), the currently published maximum amount of nucleotides cloned and introduced in a fungal expression host has not exceeded 25-28 Kbp (Yin et al. 2013). However, in the presented work we reinvigorated heterologous expression of biosynthetic pathways by combining yeast recombination and fungal transformation and succeeded to introduce an intact and functional copy of a seven gene lipopeptide producing gene cluster.

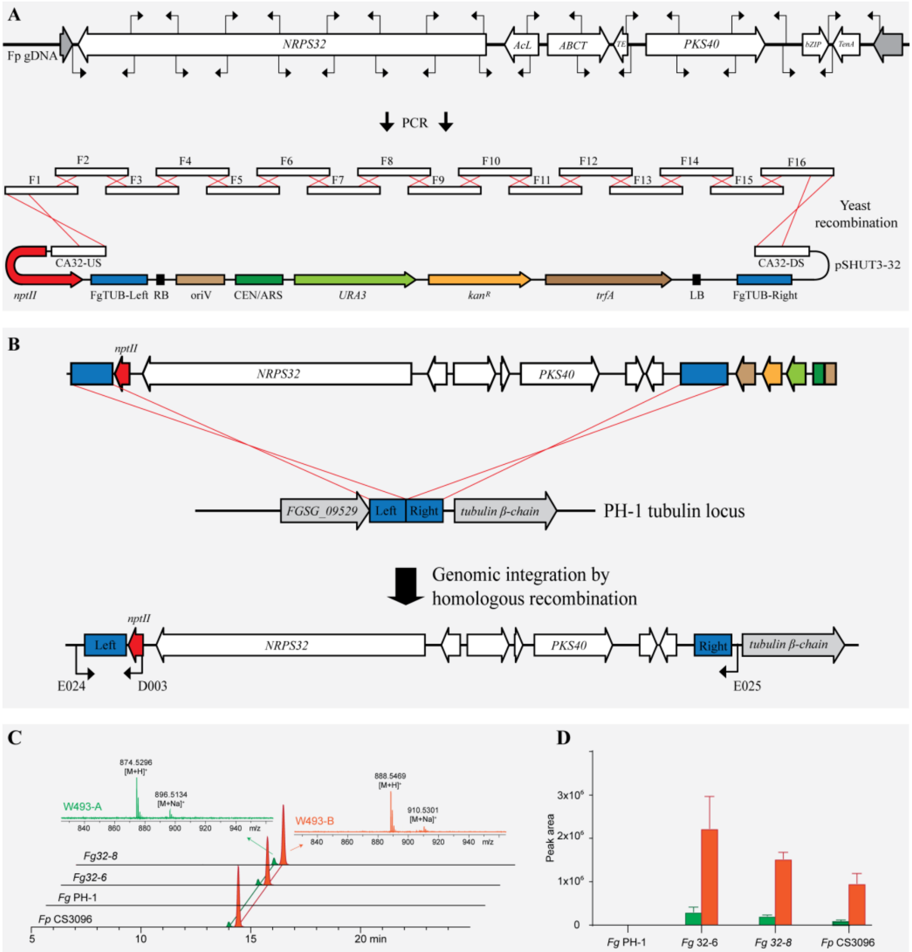

Figure 14 Workflow for reconstruction of the *W493* gene cluster in *F. graminearum* to facilitate heterologous expression. **A.** PCR-amplification of the gene cluster in 16 sections containing overlap to neighboring fragments, and assembly of the gene cluster in the pSHUT3-32 shuttle vector via yeast recombination. **B.** *W493* cluster-containing cassette integrates on the genome of *F. graminearum* via homologous recombination. The resolving genome is shown comprising all biosynthetic pathway genes. **C.** Chemical analysis of two isolated mutants against wild types *F. graminearum* (neg. control) and F. pseudograminearum (pos. control) tested for formation of the W493 A (green) and B (orange) lipopeptides. **D.** Quantification of W493 A (green) and B (orange) formation in mutants and wild types. Adapted from **Paper 3** (Nielsen et al. 2019d).

Genomic mutant DNA was purified and sequenced; showing intact copies of the gene cluster were present in two mutants. We expect with the decreasing price and increasing performance of sequencing technology, the application of Southern blot experiments could become obsolete for testing fungal mutants. To our surprise, the sequencing analysis revealed the *W493* genes had not integrated in the intended genomic position, although the construct was equipped with two ~700 bp flanking integration segments equipped to promote guided homologous recombination. Instead, mapping of the sequencing reads hinted integration in a *F. graminearum* locus orthologue to the *F. pseudograminearum* W493 gene cluster, sharing a high level of similarity to the edges of the gene cluster. Random integration may results in strain instability and loss of expression over time (Anyaogu and Mortensen 2015). However, in the context of metabolite production, mutants having several copies of the investigated gene can be considered beneficial.

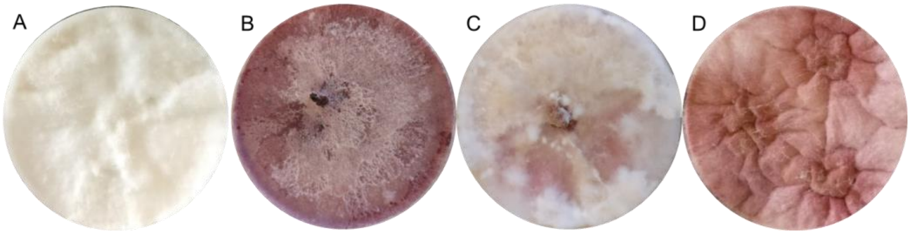

Figure 15 Fungal strains applied and prepared in **Paper 3** (Nielsen et al. 2019d). **A.** *F. pseudograminearum* CS3096 **B.** *F. graminearum* PH-1 **C.** *F. graminearum FCK* mutant *FgCK-1* **D.** *F. graminearum W493* mutant *Fg32-6*.

Chemical analysis of *W493* mutants revealed formation of the lipopeptide products W493 A and B in similar amount to the wild type *F. pseudograminearum*. However, in general, introducing intact copies of biosynthetic genes in *F. graminearum* will not guarantee expression without further modification. In the case where no metabolites were detected, researchers have previously ensured heterologous expression by overexpressing TFs or global regulator genes.

2.2.1 Outlook

The *Fusarium* metagenome comprise several predicted and uncharacterized gene clusters. We imagine the workflow presented in this paper will serve as a guide in future *Fusarium* product discovery and pathway characterization studies. The yeast assembly method can be applied to gene clusters from other fungal genera. In such cases, reevaluation of expression host would be suitable. However, the method alone is likely to not yield product titers high enough to perform structure elucidation experiments, and will excel when applied in tandem with other pathway activation strategies.

The presented workflow works for introducing gene clusters into *F. graminearum* and can be used to assert the functionality of fungal gene clusters. We applied the same strategy to the 9.9 Kbp *Fusarium presudograminearum* cytokinin (*FCK*) gene cluster. We solidified that these genes were responsible for the formation of fungal cytokinins in *F. pseudograminearum* during plant infection (**Side project 2**) (Sørensen et al. 2018). We could furthermore apply cytokinin-producing *F. graminearum* strains in plant infection assays to investigate how these metabolites contribute to plant pathogenicity (**Side project 3**) (Blum et al. 2019). Heterologous expression is therefore a welcomed tool in studies concerning both natural product discovery and fungal biology and pathogenicity.

2.3 The final piece to the polyketide pigmentation puzzle in *Fusarium solani*

The species complex *Fusarium solani* comprise several species that are regarded as important human and crop pathogens. *F. solani* separates itself from most other members of *Fusarium* by utilizing mycelial pigmentation from the *PKS3* gene cluster (fusarubins, javanicin, bostrycoidins), whereas other species use this pathway for perithecial pigmentation (Frandsen et al. 2016). The orange pigmentation of *F. solani* perithecia has been associated with *PKS35* (Graziani et al. 2004), although no identified compound(s) have been reported. Preliminary protein analysis grouped PKS35 together with the recently characterized PhnA from *Penicillium herquei* and PKS23 from the lichen forming *Endocarpon pusillum*, both produing prephenalenone as the first step in phenalenone/herquinone biosynthesis pathway (Gao et al. 2016; Harvey et al. 2018). A FAD-dependant monooxygenase phnB is responsible for the transformation of prephenalenone into phenalenone (Gao et al. 2016).

In this study I wanted to apply a mixed methods approach in order to identify and describe the *PKS35* specific products. First, the intron-less open reading frame of *PKS35* was cloned and expressed in *S. cerevisiae* confirming the first polyketide intermediate of the *PKS35* is also prephenalenone and its dehydrated product (**Figure 8 AB**). Constructing intron-less genes from all members of the cluster was deemed too troublesome. Therefore, we purchased an intron-less and codon optimized version of the phnB *F. solani* orthologue NECHADRAFT_76234, which was co-transformed into the same yeast strain. However, chemical analysis failed to reveal the formation of any novel compounds in this mutant.

In order to identify the *PKS35* pathway products, we successfully applied the overexpression system presented in **Paper 2** to activate the cluster specific transcription factor NECHADRAFT_103370. We recovered resistant mutants all displaying a deep red/orange color on growing medium (**Figure 8C**). We identified three new compounds in *F. solani* OE::*tf35* when grown on YES (candidate **1**) and Cz agar (candidate **2** and **3**). In addition, the *F. solani* OE::*tf35* mutant displayed overproduction of the pigments javanicin and bostrycoidin, hinting the *PKS3* and *PKS35* clusters are somehow co-regulated in *F. solani*.

Additionally we applied the workflow presented in **Paper 3** to clone, introduce and heterologously express the 26 Kbp gene cluster in *F. graminearum*. We identified prephenalenone, candidates **2**, **3**, and a fourth candidate (**4**) compound (**Figure 8D**). Additionally, we observed a compound matching corymbiferan lactone E, an oxo-phenalenone pigment produced in *Neonectria ditissima* (Ren et al. 2012, 2015).

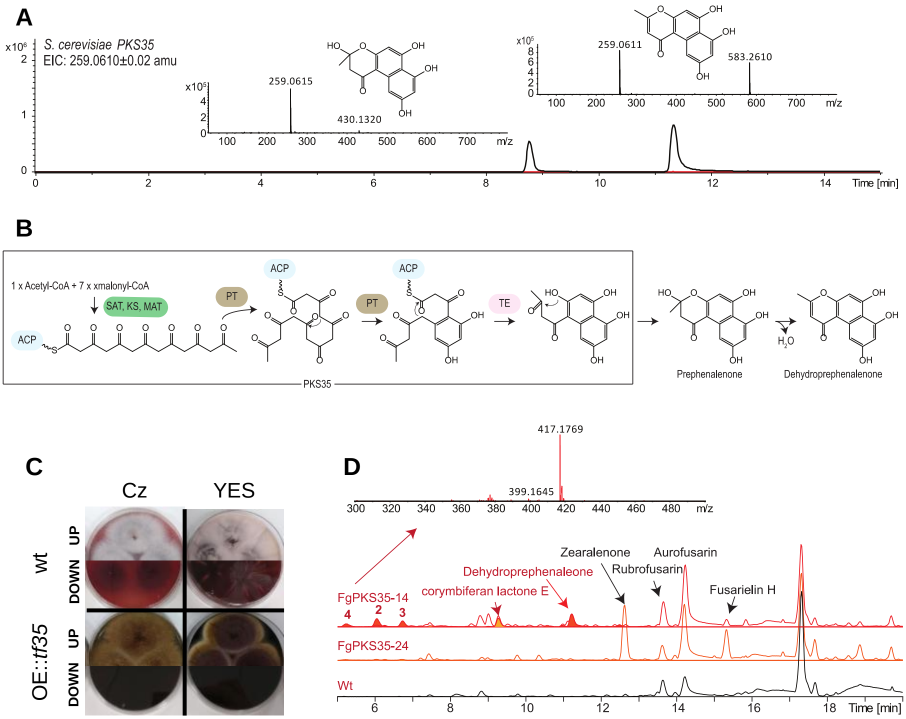

Figure 16 A. Heterologous expression of *PKS35* in *S. cerevisiae* yields prephenalenone and dehydroprehenalenone. **B.** Proposed biosynthesis pathway. **C.** *F. solani* mutants overexpressing the intrinsic transcription factor NECHADRAFT_103370 (*tf35*) from the β-Tubulin locus yields a dark red phenotype not seen for the wild type. **D.** Heterologous expression of the *PKS35* gene cluster in *F. graminearum* confirms formation of dehydroprephenalenone, candidate compounds **2**, **3** and **4** and corymbiferan lactone E. Adapted from **Paper 4** (Nielsen et al. 2019b).

This study demonstrates the power of heterologous expression of biosynthetic pathways in a product discovery setting. The compounds are currently being isolated from fungal mutants and the subsequent structural analyses will elucidate the structures of the candidate compounds and verify the identity of prephenalenone and corymbiferan lactone E, which have only been tentatively identified based on a combination of *a priori* knowledge and mass spectrometry. The ongoing chemical analysis of candidate compounds will confirm or reject their involvement in polyketide biosynthesis, and help us unravel the pigmentation puzzle of *F. solani*.

3. Conclusions & perspectives

Currently, a third of polyketides synthase and a quarter of non-ribosomal synthetase *Fusarium* genes have been linked to a specific product or product pathway. In search of novel fungal metabolites the major obstacle is attributed to transcriptional activation of gene clusters remaining cryptic. The majority previously characterized compounds are formed during growth in the laboratory, where different cultivation methods and media have been used to stimulate secondary metabolism. In order to unlock the full biosynthetic potential of these microorganisms we need to apply reverse genetic approaches such as targeted activation and heterologous expression.

In this PhD thesis my main objective was to develop and apply a vector system for introduction and expression of foreign biosynthetic pathways in the well-described plant pathogen *F. graminearum*. Yeast recombination cloning enabled rapid reconstruction of biosynthetic gene clusters and introduction to a fungal expression host was possible through a single transformation step. Large vector constructs were successfully prepared and applied to achieve heterologous expression of the 10 Kbp *F. pseudograminearum* cytokinin gene cluster, the 26 Kbp *F. solani* perithecial pigmentation cluster, and the 54 Kbp *F. pseudograminearum W493* lipopeptide producing cluster. These examples solidify the linkage between transformed gene clusters and their respective metabolite products. *Agrobacterium tumefaciens*-mediated transformation was useful for single-copy introduction of the smallest gene cluster. Attempts at introducing large gene clusters did not yield any stable transformants. Instead protoplast-mediated transformation was successfully applied for introducing and genomic introduction of large gene clusters. Genome sequencing revealed mutant genomes comprised several copies of the introduced *W493* cluster. It is generally considered beneficial to control the copy number of inserted gene cassettes, however, the aim of the project was to enable heterologous production and that was achieved in titers comparable to the origin species. This is the first report of heterologous expression of a >28 Kbp biosynthetic gene cluster paving the way for heterologous expression of the enormous and untapped non-ribosomal peptide synthase pathways. Currently more than 35 *NRPS*s have been predicted from the *Fusarium* meta-genome with no associated products (Hansen et al. 2012b, 2015). Some of these reside only in species with no current transformation protocols available such as *F. equseti* and *F. acuminatum*. Such gene clusters would be ideal targets for future heterologous expression experiments.

F. graminearum served as expression host in this study. In future studies, a host must be chosen that can express the genes in question, and thus a closely related species is a good option. The expression host in question should, for the sake of detecting recombinant pathway products, not hold any orthologues or remnants of the genes of interest. Alternatively can *S. cerevisiae* be used for rapid expression and screening of biosynthetic products, although halted by the detection and correct removal of introns (Harvey et al. 2018). An argument against using filamentous fungi as expression platforms is that *F. graminearum* has some intrinsic secondary metabolism, which might obscure the detection of recombinant pathway products. With the introduction of recyclable marker systems for *F. graminearum* (Connolly et al. 2018; Twaruschek et al. 2018), endogenous BGCs can be knocked out to ease the chemical analysis operation.

Activation of polyketide biosynthesis pathways in *F. solani* was possible by introducing overexpression of cluster-specific transcription factor genes. *S. cerevisiae* was proven to serve as a versatile platform for heterologous expression of synthases enabling rapid identification of entry compounds in polyketide synthesis. As a case study, heterologous expression in *F. graminearum* and *S. cerevisiae* together with in-host transcription factor overexpression was applied to unveil the perithecial pigmentation of *F. solani*. The first pathway intermediate prephenalenone was immediately identified. Additionally, four unknown compounds have been observed and I recently launched a structural elucidation study that aims to unravel the polyketide pigmentation puzzle of *F. solani*. The combined vector (**Paper 2**) and transformation protocol (Romans-Fuertes et al. 2016) can be applied in future studies to unlock the biosynthetic potential of *F. solani* by overexpressing biosynthetic gene or transcription factors. Furthermore, the vector system can be adapted to other transformable species of *Fusarium* by swapping the β-Tubulin locus targeting integration sequences for fragments amplified from genomic DNA. Not all species of Fusarium have associated ATMT protocols, however the insertion cassette can be PCR-amplified from the vector and transformed into protoplasts, if applicable. An obvious continuation of this study will be to investigate the bioactivity of isolated candidate compounds. Previously, scientists from our institute screened *Fusarium* metabolites for antimicrobial activity by applying a fast digital time-lapse microscopy method (Sondergaard et al. 2016).

During the PhD I participated in two side projects where cytokinin producing *F. graminearum* mutants were included in the investigation of the functional role of fungal cytokinins during plant infection, demonstrating the broad application of the presented vector system in fungal biology.

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