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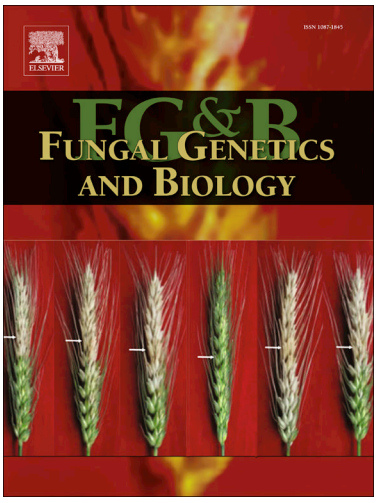

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Heterologous Expression of Intact Biosynthetic Gene Clusters in *Fusarium graminearum*

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Abstract

Filamentous fungi such as species from the genus *Fusarium* are capable of producing a wide palette of interesting metabolites relevant to health, agriculture and biotechnology. Secondary metabolites are formed from large synthase/synthetase enzymes often encoded in gene clusters containing additional enzymes cooperating in the metabolite's biosynthesis. The true potential of fungal metabolomes remain untapped as the majority of secondary metabolite gene clusters are silent under standard laboratory growth conditions. One way to achieve expression of biosynthetic pathways is to clone the responsible genes and express them in a well-suited heterologous host, which poses a challenge since *Fusarium* polyketide synthase and non-ribosomal peptide synthetase gene clusters can be large (e.g. as large as 80 kb) and comprise several genes necessary for product formation. The major challenge associated with heterologous expression of fungal biosynthesis pathways is thus handling and cloning large DNA sequences. In this paper we present the successful workflow for cloning, reconstruction and heterologous production of two previously characterized *Fusarium pseudograminearum* natural product pathways in *Fusarium graminearum*. *In vivo* yeast recombination enabled rapid assembly of the *W493* (*NRPS32-PKS40*) and the *Fusarium Cytokinin* gene clusters. *F. graminearum* transformants were obtained through protoplast-mediated and *Agrobacterium tumefaciens*-mediated transformation. Whole genome sequencing revealed isolation of transformants carrying intact copies the gene clusters was possible. Known *Fusarium cytokinin* metabolites; fusatin, 8-oxo-fusatin, 8-oxo-isopentenyladenine, fusatinic acid together with cis– and trans-zeatin were detected by liquid chromatography and mass spectrometry, which confirmed gene functionality in *F. graminearum*. In addition the non-ribosomal lipopeptide products W493 A and B was heterologously produced in similar amounts to that observed in the *F. pseudograminearum*

doner. The *Fusarium* pan-genome comprises more than 60 uncharacterized putative secondary metabolite gene clusters. We nominate the well-characterized *F. graminearum* as a heterologous expression platform for *Fusarium* secondary metabolite gene clusters, and present our experience cloning and introducing gene clusters into this species. We expect the presented methods will inspire future endevours in heterologous production of *Fusarium* metabolites and potentially aid the production and characterization of novel natural products.

Keywords: Secondary metabolites, *Fusarium*, heterologous expression, non-ribosomal peptides, lipopeptides, cytokinin

1 Introduction

Polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) are enzymes responsible for producing some of the most interesting secondary metabolites (SMs) in relation to health and agricultural potential (Marahiel, 2009; Staunton and Weissman, 2001). Often the polyketide or non-ribosomal peptide products are further modified by tailoring enzymes, such as cytochrome P450 monooxygenases and methyltransferases. Fungal SM gene clusters may encode transcription factors, specific precursor synthases, transporters and/or a self-protection mechanism, all important for the formation of the final product (Yu and Keller, 2005). The environmental conditions triggering secondary metabolism gene expression often are unpredictible and the majority of genes are not expressed in standard laboratory growth medium (Brakhage, 2013; Janevska and Tudzynski, 2018). One explaination has been that a major portion of SM clusters reside within non-conserved chromosomal regions which overlap with tri-methylation of lysine 27 in histone 3 (H3K27me3) enriched regions, defined as heterochromatin, associated with low gene expression levels (Connolly et al., 2013; Reyes-Dominguez et al., 2012; Zhao et al., 2014). This biological phenomenon has contributed to the classification of uncharacterized clusters that show little to no expression under laboratory conditions, as ‘silent’ or ‘cryptic’ gene clusters. This problem has spawned many endeavors in developing tools for gene cluster activation and SM-gene cluster linking, including overexpression of key synthases/synthetases or manipulation of local and global regulators (Katz and Baltz, 2016; Keller, 2019; Wiemann and Keller, 2014).

In order to achieve expression of the majority of fungal SMs, SM gene clusters can also be moved to an alternative host for heterologous expression, which present many advantages including tools to enhance production through further genetic manipulation and pathway elucidation (Alberti et al., 2017; Boecker et al., 2018). For instance, heterologous expression of fungal biosynthetic genes in *Saccharomyces cerevisiae* has proven a feasible avenue for novel metabolite discovery (Harvey et al., 2018; Tsunematsu et al., 2013).

The first case of heterologous reconstruction of a multi-enzyme gene cluster was the PKS-NRPS tenellin cluster in the well-characterized filamentous fungus *Aspergillus oryzae* (Heneghan et al., 2010). Since then, a handful of cases (Alberti et al., 2017) have been reported using similar workflows (Anyaogu and Mortensen, 2015) in which members of the target gene cluster are predicted from DNA, using bioinformatics tools such as AntiSMASH (Weber et al., 2015). Initially, the target genes are cloned into either a cosmid/fosmid library (Sakai et al., 2012; Smith et al., 1990) or amplified through PCR and assembled into vectors using USER fusion (Nielsen et al., 2013), Gibson assembly (Schuetze and Meyer, 2017) or yeast mediated recombinatorial assembly recombination (Yin et al., 2013). Recently, the development and application of Fungal Artificial Chromosomes has also been reported for this purpose (Clevenger et al., 2017). Secondly the genes are introduced into a suitable expression host such as *Aspergillus oryzae* or *Aspergillus nidulans* (Anyaogu and Mortensen, 2015). These Aspergilli are often modified strains deficient of non-homologous end joining (Nayak et al., 2006), because targeted genomic integration is preferred over random integration to ensure stability of foreign genes (Chiang et al., 2013; Hansen et al., 2011; Mikkelsen et al., 2012). Furthermore, random genomic insertions may disrupt endogenous genes and the genomic position can influence whether acquired genes attain functionality (Husnik and McCutcheon, 2017). The toolbox for heterologous expression in Aspergilli has expanded rapidly in the last decade with the development and implementation of novel methods such as polycistronic gene expression and cleavage peptide signals (Hoefgen et al., 2018; Schuetze and Meyer, 2017).

In recent years, sequencing of fungal genomes has been booming and recently 67 different polyketide synthase genes and 52 non-ribosomal peptide syntethase genes have been observed across 31 *Fusarium* genomes (Brown and Proctor, 2016; Hansen et al., 2015). Some clusters are shared within the genus (e.g. *PKS3* responsible for formation of the pigments fusarubin and bostrycoidin) while others are unique to a single species (e.g. *NRPS32-PKS40* responsible for the formation of the W493 lipopeptides, only found in *F. pseudograminearum*). The largest detected SM gene in *Fusarium* contains the 40 Kb *NRPS34,* which resides in

an 82 kb gene cluster. Some of the metabolites produced by *Fusarium* are of relevance to food and feed safety, e.g. fumonisin B1 (*PKS24*) which is a harmful mycotoxin (Voss and Riley, 2013), while other compounds such as sansalvamide (*NRPS30*) and apicidin (*NRPS31*) are potential drug candidates (Niehaus et al., 2014; Romans-Fuertes et al., 2016). Roughly one-third of *Fusarium PKS*-containing clusters and one quarter of the *NRPS*-containing clusters have been linked to a corresponding SM or biosynthetic pathway (Nielsen et al., 2019). The *Fusarium* pan-genome is thus a treasure chest of undiscovered metabolites (Hoogendoorn et al., 2018), and heterologous expression might present itself as the key to unlock a wealth of chemical diversity and bioactive compounds. We chose the well-characterized *F. graminerarum* for heterologous expression of *Fusarium* biosynthetic gene clusters, as good expression can generally be expected in a closely related species. *Fusarium graminearum* provides several benefits to this purpose such as a efficient gene targeting via homologous recombination (Frandsen et al., 2012; Twaruschek et al., 2018), several developed transformation and gene editing techniques (Connolly et al., 2018; Gardiner and Kazan, 2018), together with a well characterized intrinsic secondary metabolism (Bahadoor et al., 2018; Frandsen et al., 2016; J. L. Sørensen et al., 2014; Westphal et al., 2018a; Wollenberg et al., 2017).

Previously, we reported the reconstruction and successful heterologous expression of a four gene 10 kb cluster from *F. pseudograminearum* (Sørensen et al., 2018). In this paper, we present our experience with cloning, introducing and heterologously expressing SM clusters of variable size in *F. graminearum* and discuss the limitations and benefits of heterologous production as a tool for SM discovery. In this paper we also present the first case of heterologous expression of an intact *Fusarium* NRPS gene cluster in a filamentous fungal host. The presented methods will serve as a much needed set of tools that could enable activation and pathway elucidation of natural products from *Fusarium*.

2. Materials and methods

2.1 Strains and media

Fusarium Cytokinin (*FCK*) (FPSE_06371–20002) and *W493* (*NRPS32-PKS40*) (FPSE_09183–09189) gene clusters were PCR amplified from *F. pseudograminearum* (*Fp*) CS3096 genomic DNA (Gardiner et al., 2012). The host for heterologous expression of SM clusters was performed in *F. graminearum* (*Fg*) (PH-1; NRRL 31084) (Trail and Common, 2000). The fungi were grown, maintained and selected on Czapek-Dox [35 g/L Cz broth (Sigma C1551), 1 mL /L trace metals solution (0.1 g/L $ZnSO_4 \cdot 7H_2O$ and 0.05 g/L $CuSO_4 \cdot 5H_2O$)] medium or YPG [1% yeast extract, 2% peptone, 2% D-glucose] medium with 300 µg/mL G418 geneticin sulfate (Gibco 11811031), and 2% agar for solid medium. Fungi were grown at 24-26˚C in darkness unless otherwise specified. Production of *F. graminearum* macroconidia was done by inoculating 70 mL carboxymethyl cellulose medium (Cappellini and Peterson, 1965) with 4-8 × 5mm^2 agar plugs cut from a 7 day old PDA (potato dextrose agar, Sigma 70139) plate and shaking at 19°C, 150 rpm, for 3-5 days. Macroconidia were filtered through a sterile glass wool-filled syringe, centrifuged at 3000 g, resuspended in sterile H_2O and kept at 4˚C. To induce secondary metabolite production fungi were cultivated on yeast extract sucrose medium (YES, Scharlau yeast extract, Barcelona, Spain) (Sørensen and Sondergaard, 2014).

Escherichia coli DH5α was used for plasmid propagation and yeast-plasmid recovery. For *E. coli* growth and selection, we applied solid (2% agar) or liquid Luria-Bertani (LB, Lennox) amended with 25 µg/mL kanamycin when applicable.

Agrobacterium tumefaciens LBA4404 was used during *Agrobacterium tumefaciens*-mediated transformation of *Fusarium*. *A. tumefaciens* was grown in either LB or YM [0.4 g/L yeast extract, 10 g/L mannitol, 0.1 g/L NaCl, 0.1 g $MgSO_4$, 0.5 g/L K_2HPO_4 $3 \cdot H_2O$, pH 7], added antibiotics 10 µg/mL rifampicin, 100 µg/mL streptomycin, and when appropriate 25 µg/mL kanamycin.

Saccharomyces cerevisiae BY4743 (Euroscarf Y20000) was used for plasmid assembly and cloning of fungal DNA. Growth and selection of yeast was carried out using yeast synthetic dropout medium without uracil (SC-U; Sigma Y1501) prepared according to manufacturers instructions with yeast nitrogen base (Sigma, Y0626).

2.2 Cluster orthology analyses

To analyse the introduced gene clusters in the expression host the nucleotide sequences were compared using BLAST and blastx (blast.ncbi.nlm.nih.gov) and generated linear comparison maps of loci with EasyFig v. 2.1 (Sullivan et al., 2011). Genbank flat files from *Fg* (Chr3; CM000576.1 (Cuomo et al., 2007)) and *Fp* (Chr1; CM003198.1, Chr3; CM003200.1 (Gardiner et al., 2012)) were loaded into EasyFig and comparison maps were generated using 70 bp minimum alignment length and 0.35 minimum identity score.

2.3 PCR amplification of fungal biosynthetic gene clusters.

Genomic DNA was extracted from fresh mycelium using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Primers (**Supplementary Table 1-2**) for PCR amplification of gene clusters were designed using the Primer3Plus software (Untergasser et al., 2007) based on available *Fp* genomic sequence (Gardiner et al., 2012). The parameters were set to identify primers producing 3-4 kb products with at least 50 bp of homology to neighboring PCR product or the shuttle vector. Genes included in constructs were based on previous gene cluster characterization; *FCK* (Sørensen et al., 2018), *W493* (J. L. Sørensen et al., 2014). Full gene clusters were PCR-amplified in 3168-3941 bp segments with at least 59 bp neighboring overlap using the Phusion HS II DNA polymerase (Thermo Fisher scientific) following manufacturer's instructions. For amplification of the *FCK* cluster, the two outermost primers had a 36 bp tail homologous to shuttle vector's multiple cloning site.

Before reconstruction of gene clusters in yeast PCR fragments were quantified using a Nanodrop 2000C (Thermo Fisher Scientific) and pooled to equalize the molar concentration of different fragments. The pooled

FCK PCR products (CK1-3) were purified using the QIAquick PCR Purification Kit (Qiagen) and the pool contained 40-80 fmol of the three fragments. Pooled *W493* PCR products (C1-16) were concentrated and purified by using the QIAquick PCR Purification Kit (QIAquick PCR Purification Kit, QIAGEN). The *W493* concentrated PCR product pool contained approximately 200-230 fmol of each fragment.

2.4 Construction and validation of gene cluster containing vectors

Two shuttle vectors pSHUT1 and pSHUT3 were prepared by modifying the in-house *A. tumefaciens* shuttle vector U-GOTL (Josefsen et al., 2012) (**Supplementary Table 3**). These shuttle vectors contain the following elements: An *Agrobacterium* T-DNA cassette comprising a multiple cloning site and *nptII* selection marker in between two segments with homology to the *F. graminearum* β-Tubulin locus*; FgTUB-Left and FgTUB-Right* (Josefsen et al., 2012). The vector backbones also contained bacterial replication elements *trfA*, *IncP*, antibiotic resistance (*Kan*^{R}), auxotrophic selection marker *URA3,* and either *2μ* or CEN6/ARSH4 yeast origins of replication. For pSHUT1 the replication origin was the *2μ* element from pYES2 (Invitrogen) (**Supplementary Figure S1A**). For pSHUT3 the 2μ origin was replaced with the low copy number CEN6/ARSH4 yeast origin of replication from pRS315 (ATCC®77144) (**Supplementary Figure S1B**). The pSHUT3 plasmid was further modified for reconstructing the *Fp W493* cluster by inserting two 1 kb segments homologous to cluster flanking regions separated by a *SmaI* restriction site inside the multiple cloning site.

Competent *S. cerevisiae* cells were transformed (Gietz and Schiestl, 2007) with concentrated pools of SM gene clusters containing PCR fragments together with linearized shuttle vector DNA purified with QIAquick Gel Purification Kit (**Table 1**). Gene clusters were assembled in vectors through transformation-associated homologous recombination between PCR fragments and linearized vector in yeast (**Figure 1A**). Transformed yeast cells were selected on SC-U and grown for 2 days at 30°C before colonies were streaked on fresh SC-U plates and incubated over night at 30°C. Routinely, we performed yeast colony PCR to verify the cloning as

previously described (Kouprina and Larionov, 2008). Vector assemblies were recovered by isolating yeast vector DNA following procedure applied in (Noskov et al., 2011). Precipitated plasmid DNA was resuspended in 300 µL H_2O and pipetted vigorously to resuspend. 1 µL yeast vector DNA was mixed with 40 µL electrocompetent *E. coli* cells and transformed using the MicroPulser™ Electroporation apparatus (Bio-Rad, Hercules, CA, USA) in a 1 mm wide electrocuvette using the *Ec1* setting. Transformed *E. coli* were selected on LB kanamycin plates and incubated at 37°C overnight. Single colony isolates were streaked on fresh LBA kanamycin.

Gene cluster carrying vectors were purified from LB kanamycin cultures using the QIAprep Spin Miniprep Kit or QIAGEN Plasmid Midi Kit (QIAGEN). Diagnostic PCR was performed on individual yeast clones across homologous recombination events between different PCR fragments and between PCR fragments and shuttle vector (Primers are listed in **Supplementary table T4-5**). Restriction enzyme digest was performed to further validate yeast recombination constructs, following the manufacturer's instructions (NEB, Thermo Scientific).

2.5 *Agrobacterium tumefaciens*-mediated transformation

Vector constructs holding fungal gene cluster DNA was introduced into *A. tumefaciens* strain LBA4404 by electroporation (Lin, 1995) using the *Agr* setting of the Micropulser™ Electroporation apparatus (Biorad, Hercules CA, USA). *F. graminearum* macroconidia were transformed by ATMT as described previously (Malz et al., 2005), without pre-germinating fungal spores as suggested by (Frandsen et al., 2012). *At* strains were grown in liquid culture to an OD_{600} ≈ 0.7. *F. graminearum* was co-cultivated with *At* strains LBA4404::pSHUT1:CK, LBA4404::pSHUT3:32-06, LBA4404::pSHUT3:32-08, and LBA4404::pSHUT3-32 on induction medium [40 mM MES, 0.2 mM acetosyringone, 0.2% glucose, 0.5% glycerol, 11 mM KH_2PO_4, 12 mM K_2HPO_4, 2.6 mM NaCl, 2 mM $MgSO_4 \cdot 7H_2O$, 0.44 mM $CaCl_2 \cdot 2H_2O$, 0.01 mM $FeSO_4 \cdot 7H_2O$, 3.8 mM $(NH_4)_2SO_4$] for 50 hours at 25°C in darkness.

Transformed fungi were selected as described by (Frandsen et al., 2012) using 300 µg/mL cefoxitin sodium salt (Sigma) and 150 µg/mL G418.

2.6 Protoplast transformation

Plasmids pSHUT3, pSHUT3:32-06, and pSHUT3:32-08 were propagated in *E. coli* and isolated in high concentration following the procedures described in (Pronobis et al., 2016) using reagents from QIAprep Spin Miniprep Kit (QIAGEN). Prior to fungal transformation plasmid DNA was linearized with restriction enzymes cutting inside the plasmid backbone and not the fungal DNA, unless otherwise specified. 100 mL yeast extract peptone dextrose (YEPD) medium [0.3% yeast extract, 1% peptone , 2% D-glucose] was inoculated with 4×10^6 fresh conidia and incubated with shaking 150 rpm at 29°C for 14-16 hours. Protoplasts were prepared as described elsewhere (Gaffoor et al., 2005) and washed, transformed, regenerated and overlayed as described previously (Twaruschek et al., 2018; Varga et al., 2015). Putative mutants appeared after 4-6 days. Transformants were streaked onto solid Czapek-Dox media containing 300 µg/mL G418.

2.7 Fungal mutant screening

Colony PCR was routinely used to test isolated fungal transformants. Antibiotic resistant transfromants were incubated on PDA plates for 3-4 days. With a sterile pipette tip, a minute amount of fresh hyphial mycelium was resuspended in 300 µL fungal lysis buffer [0.2 M NaCl, 0.1 % Triton-X100, 0.2 % SDS, 10 mM Tris-HCl, 50 mM EDTA, pH 7.5]. The sample was vortexed vigorously for one minute and centrifuged for one minute at 10.000 g. 1 µL of the supernatant was used in PCR [0.005 U/µL Phusion II HS Polymerase, 1 x HF buffer, 200 µM dNTP, 0.5 µM primer1, 0.5 µM primer2, 2 mg/mL BSA]. Primers used in fungal colony PCR are listed in supplementary table T8.

2.8 Sequencing of fungal transformants

From a week-old YPG plate mycelium was scraped with a sterile toothpick and submerged into fresh YPG pH 6.5 culture medium. The mycelium was incubated at 28°C, 100 rpm for 72 hours before harvesting by filtering through a layer of sterile miracloth and washed in sterile water. The mycelium was sqeezed to remove excess liquid and lyophilized over night.

Fungal tissue samples were sent to DNAsense APS (Aalborg, Denmark) for DNA purification and Nanopore sequencing services utilizing the miniON flow cell system (Oxford Nanopore Technologies, UK). Following import of the trimmed and quality-filtered reads into CLC Genomics Workbench v. 12 (CLC Bio, Qiagen, Denmark), reads were mapped to the *F. graminearum* PH-1 reference genome (chromosome 1,2,3 and 4) and to the (theoretical) *in silico* generated chromosome of each transformant (chromosome 4, β-Tubulin locus).

2.9 Heterologous expression and metabolite analysis

Function of introduced gene clusters was tested by inoculating plates of YES agar and incubating them for 14 days. Secondary metabolites were extracted from solid agar plugs submerged in organic extraction mix ethylacetate:dichlormethane:methanol (3:2:1) with 1% formic acid in an ultrasonic bath for 1 hour (Smedsgaard, 1997). The solvent was transferred to a clean glass vial and was evaporated under a flow of nitrogen gas at 40°C. Dried samples were resuspended in 600 µL of methanol and centrifuged for 2 minutes to remove particulate impurities. The supernatants were then transferred to HPLC vials and analyzed by high performance liquid chromatography (Hitachi Elite LaChrom HPLC, Hitachi, Tokyo, Japan) coupled to a high-resolution mass spectrometer (HRMS; Bruker compact MS ESI-Q-TOF, Bruker Daltonics, Bremen, Germany) operating in positive ionization mode. 10 µL of each extract was injected and separated on a C6-phenyl column (150 × 4.6 mm Ascentis Xpress 2.7 µm, Sigma-Aldrich, St. Louis, MO, USA) as previously described (Westphal et al., 2018b). Cytokinins and W493 A and B were detected using the extraction ion chromatograms based on

previous studies (J. L. Sørensen et al., 2014; Sørensen et al., 2018). The expected and observed protonated ions are listed in **Supplementary Table 6**.

3 Results

3.1 Target biosynthetic gene clusters

To explore the potential of *F. graminearum* as a host for heterologous production of secondary metabolites we chose two characterized biosynthetic gene clusters of different size. The locus chosen for targeted integration resides in a euchromatic region near the β-Tubulin gene, in theory enabling expression of foreign genes. As an example of a small gene cluster, we chose the *FCK* cluster from *F. pseudograminearum*. The gene cluster has a size of 9.9 kb and contains four genes, *FCK1* (fusatin synthase), *FCK2* (cytochrome P450), *FCK3* (putative glycosyl transferase), and *FCK4* (alcohol acetyltransferase) (Sørensen et al., 2018). In culture, *F. pseudograminearum* produces four fungal cytokinins: Fusatin, 8-oxo-fusatin, fusatinic acid, and 8-oxo-isopentenyladenine (**Figure 2A**). Comparison of *F. graminearum* and *F. pseudograminearum* showed that the *FCK* gene cluster is present only in *F. pseudograminearum*, while neighboring genes in *F. pseudograminearum* (FPSE_6370 and FPSE_06373) had high identity to FGSG_13984 and FGSG_11345 (**Figure 2C**).

As an example of a large biosynthetic gene cluster, we chose the 54 kb *W493* gene cluster from *F. pseudograminearum* (J. L. Sørensen et al., 2014). In addition to the *PKS40* and *NRPS32*, this cluster contains five genes and is responsible for production of the two lipopeptides W493-A and B (**Figure 2B**). This gene cluster is absent in *F. graminearum* although the borders have minimal homology to a locus on *F. graminearum* chromosome 1 (**Figure 2D**).

3.2 Amplification and cloning of target gene clusters

The targeted gene clusters were amplified in 3-4 kb PCR fragments with at least 50 bp of overlap between adjacent products. Following this strategy, the *FCK* cluster was amplified in three PCR fragments and the *W493* cluster in 16 fragments (**Figure 1A**).

The purified PCR fragments were pooled and assembled by yeast homologous recombination with a linearized shuttle vector. The three fragments covering the *FCK* gene cluster were cloned into the vector pSHUT1, which contains the high copy *2μ* origin of replication. The resulting plasmid was amplified in *E. coli* and correct assembly of the 19.238 bp plasmid was verified by PCR across recombination junctions and by *Pst*I restriction enzyme digest (**Supplementary Figure S2**).

The 16 PCR fragments covering the *W493* gene cluster were assembled into the modified version of pSHUT3; pSHUT3-32, which contain two 1-kb segments homologous to border sites of the gene cluster to, theoretically, increase cloning success. 64 isolated yeast transformants were analyzed by colony PCR with primer sets D002/D003 and D022/D023 (**Supplementary Table T7);** 58 out of 64 (91 %) yeast colonies produced a band confirming the presence of the *nptII* marker, and 53 (83 %) produced the E8 band confirming presence of gene cluster DNA (data not shown). Accurate recombination of the 63.101 bp plasmid for two randomly selected and isolated plasmids (pSHUT3:32-06, pSHUT3:32-08) (**Figure 1C**) was confirmed with 17 diagnostic PCRs and restriction enzyme digest analysis (**Supplementary Figure S3**), and sequencing from backbone into gene cluster DNA (not shown).

3.3 Heterologous production of cytokinins in *F. graminearum*

The *FCK* gene cluster was introduced in a non-coding region of *F. graminearum* downstream the β-Tubulin locus (FGSG_09530) through ATMT. We obtained G418-resistant transformants at a frequency of 2.3 colonies per 10^6 spores, which were subcultured and subject to colony PCR to confirm presence of the *nptII* marker gene (**Supplementary Figure S4**). One transformant, *FgCK-1*, was chosen for further analyses. Genomic DNA was isolated from *FgCK-1* and full genome sequencing confirmed presence of intact and correctly integrated *FCK* gene cluster (**Figure 3A and B**).

The strain was grown on YES medium together with the wild type for 14 days in the dark at 25°C, before the production of secondary metabolite was analyzed by HPLC-HRMS (**Supplementary Figure S5A**). The analyses showed that cytokinins could only be detected in *FgCK-1* and not in the parental strain (**Figure 3C**). The *FgCK-1* transformant was able to produce the four *F. pseudograminearum* cytokinins fusatin, 8-oxo-fusatin, 8-oxo-isopentenyladenine, fusatinic acid together with the more commonly observed cis- and trans-zeatin (Sørensen et al., 2018).

3.4 Heterologous production of W493 A and B in *F. graminearum*

The ATMT protocol was also initially attempted for transferring the *W493* gene cluster into *F. graminearum*. However, repeated attempts failed to produce resistant transformants and we therefore switched to protoplast transformation. Two isolated yeast constructs pSHUT3:32-06 and pSHUT3:32-08 carrying intact copies of the *W493* gene cluster were linearized and used to transform *F. graminearum* protoplasts. We obtained one pSHUT3:32-06 transformant and one pSHUT3:32-08 transformant, named *Fg32-6* and *Fg32-8*, respectively. Whole-genome sequencing of transformants *Fg32-6* and *Fg32-8* confirmed the presence of the *W493* gene cluster cassette in both transformants (**Supplementary Figure S6**). However, in both transformants, the cassettes had not integrated in the intended β-Tubulin locus and manual read coverage analysis further suggested multiple insertion events in the transformants recovered from protoplast-mediated transformation (**Figure 4A and B)**. In order to find potential ectopic interation positions, filtered sequencing reads containing coverage to the *W493* gene cluster were mapped to *F. graminearum* genome (**Supplementary Figure S7**). Both transformants contained reads mapping to positions 6.461.186–6.465.555 on chromosome 1, surrounding FGSG_12038. This is the exact position that the *W493* gene cluster appears to reside in the *F. pseudograminearum* genome relative to *F. graminearum* (**Figure 2D**). We identified two segments sharing high similarity between the borders of the *W493* insertional cassette and the *F. graminearum* FGSG_12038 locus:

Left end of amplified cluster (1.727 bp) 85% identity, and right end of amplified cluster (1.858 bp) 94% identity which may have guided homologous integration of the *W493* cassette in the FGSG_12038 locus, outcompeting the likelihood of integration in the β-Tubulin locus (**Figure 1D**). *Fg32-6* sequencing reads mapped to one additional position near FGSG_08443–08448 on chromosome 2, and *Fg32-8* mapped to two positions near FGSG_10045 and FGSG_09529–09530 on chromosomes 1 and 4. The latter position being the β-Tubulin locus, however, diagnostic PCR with primers E024/E025 (**Figure 1E**) yielded a band identical to the reaction performed on *F. graminearum* wild type DNA (not shown), ruling out a potential insertion event in the β-Tubulin locus in *Fg32-8*. Furthermore, filtered sequencing reads that mapped to the *W493* gene cluster also included pSHUT3 backbone elements, suggesting both transformants had experienced integration of the vector backbone (**Supplementary Figure S8**). Open reading frames, terminator and promoter sequences of seven introduced genes in *Fg32-6* were identical to *F. pseudograminearum* genomic reference (CM003200.1). A single discrepancy was observed in the ABC Transporter (FPSE_09185) of *Fg32-8* displaying a putative substitution event resulting in the G1096D mutation.

Secondary metabolite analyses (**Supplementary Figure S5B**) of the two transformants showed that they were both able to produce W493 A and B, which was not observed in the wild type *F. graminearum* (**Figure 4C**). The levels of both compounds was to that observed in the *F. pseudograminearum* donor strain (**Figure 4D**). This observation comfirms all *W493* pathway genes were integrated in the *F. graminearum* genome and were fully functional in both transformants. Since the two transformants *Fg32-6* and *Fg32-8* were constructed using two separate yeast recombination constructs (pSHUT3:32-06, pSHUT3:32-08), we assume PCR amplification and yeast recombination is a robust cloning tool for assembly of large intact gene clusters.

4. Discussion

We set out to develop a system for moving full size gene clusters into the well characterized *F. graminearum*. Cloning of biosynthetic gene clusters were based on yeast-mediated assembly of PCR-amplified segments into a linearized backbone including an auxotrophic selection marker. For constructs larger than 20 kb we found exchanging the origin of replication to CEN/ARS aided in yeast-mediated cloning success, which solves size limitation bottleneck when constructing large vectors for genomic integration in filamentous fungi. Others have overcome this problem by splitting the target clusters in two and transforming host organisms using multiple iterations and recyclable markers (Chiang et al., 2013; Nielsen et al., 2013; Yin et al., 2013). We conclude the application of yeast-mediated recombinatorial cloning is suitable reconstructing small and large fungal biosynthetic gene clusters, as it allows for construction of plasmids including all pathway genes that can be introduced into the fungal host in a single transformation.

Our initial intent was to introduce gene cluster DNA into *Fg* via ATMT (Frandsen et al., 2012). Consequently, the pSHUT backbone comprises elements from pBI121, including the low copy number origin of replication IncP providing stable replication of large plasmids in both *E. coli* and *Agrobacterium* (Komari et al., 2006). ATMT is a robust transformation tool that has served as the standard in several *Fusarium* studies (Romans-Fuertes et al., 2016; L. Q. Sørensen et al., 2014). In our study, ATMT was successfully applied for introducing the smaller *FCK* gene cluster, on the contrary the transformation of the larger *W493* cluster was only possible through PMT. However, similar observations has suggested a decreasing transformation efficiency with larger T-DNA inserts, and although one study reported introduction of up to 75 kb T-DNA inserts in *F. oxysporum* f. sp. *lycopersici* via ATMT, their efforts yielded few antibiotic resistant transformants which did not harbour intact T-DNA inserts (Takken et al., 2004).

Some strains of *Agrobacterium* have been reported be unsuitable to stably carry large plasmids (Song et al., 2003). To investigate this problem *Agrobacterium* strains were maintained on selective LB medium containing kanamycin. Plasmid DNA isolated from LBA4404::pSHUT3:32-06 and LBA4404::pSHUT3:32-08 were analyzed with PCR using the same primers as used for confirming correct recombination assembly of plasmids in yeast (data not shown). PCR analysis verified plasmid integrity in *Agrobacterium* strains. However, as we did not obtain any transformants with pSHUT3:32 (T-DNA; 57.776 bp) we propose the existence of an undefined upper microphysical size limitation for T-DNA integration in *F. graminearum*.

In an attempt to optimize the ATMT protocol, we changed the acetosyringone concentration during the *Agrobacterium* virulence factor-induction and *Agrobacterium*-fungal co-cultivation steps. We found increasing acetosyringone concentration to 500 µM in these two steps resulted in a significant increase in number of resistant mutants appearing per plate (**Supplementary Table T8**). 10 randomly selected *Fg*::pSHUT3-32 (empty vector) isolates were analyzed by fungal colony PCR. 9 out of 10 colonies displayed recombination between vector and the target locus (data not shown). However, this modification did not aid the transformation with the large gene cluster. In conclusion, ATMT serves as a robust tool resulting in single integration events (Idnurm et al., 2017) suitable for smaller modifications in *Fusarium*, such as promoter swapping (Wollenberg et al., 2017) or introduction and heterologous expression of smaller biosynthetic genes.

Transformation with the larger *W493* gene cluster was only possible through protoplast transformation. Protoplasting is used in *Fusarium* studies (Connolly et al., 2018), although ATMT has been proposed to be a better method (Idnurm et al., 2017). The need for high amounts of plasmid DNA creates a bottleneck, which poses a problem since larger constructs need to carry a low copy origin of replication in order to facilitate propagation in *E. coli*. However, sufficient plasmid can be obtained with generic plasmid isolation kits from large culture volumes or by pooling several preps. Furthermore we recommend linearizing plasmid DNA with

restriction endonucleases cutting inside the plasmid backbone to promote increase in yield of recovered PMT transformants (**Supplementary Table T9**). Another disadvantage in the protoplast transformation is the risk of ectopic or multiple integration events, as observed in the *Fg32-6* and *Fg32-8* transformants.This could be caused by the flanking FgTUB-Left and FgTUB-Right regions (700 bp) being too short to promote targeted recombination. In other studies reasearchers have used homology segments up to 1000-1500 bp in size (Bahadoor et al., 2018; Frandsen et al., 2012), whilst other have used as little as 500 bp (Gaffoor et al., 2005). In addition, genome sequencing hinted the *W493* cluster had, in both transformants, recombined with the FGSG_12038 locus to which the gene cluster ends exert high sequence similarity. To our surprise, the gene cluster could have integrated in the FGSG_12038 locus through homologous recombination.

Heterologous expression is potentially the most universal strategy to unlock the biochemical potential of silent gene clusters (Clevenger et al., 2017; Kakule et al., 2015). According to genomic analyses, >35 *NRPS* clusters are uncharacterized within the *Fusarium* pan-genome (Hansen et al., 2015), underlining the potential of heterologous expression strategies, such as this work, as an avenue for tapping into novel chemical diversity. Single gene PKS (Fujii et al., 1996; Hansen et al., 2011), NRPS (Boecker et al., 2018; Brandenburger et al., 2017; Geib et al., 2019) and hybrid synthases/synthetases (Munawar et al., 2013) have previsouly been heterologously expressed successfully in filamentous fungi and yeasts, predominantly in the species *A. oryzae*, *A nidulans* and *S. cerevisiae*. Yeast expression provides a high-throughput platform for activation of silent biosynthetic genes (Harvey et al., 2018), however, correct intron splicing of fungal genes, the requirement of specialized compartmentalization, and the absence of rare substrates creates challenges that can halt the metabolite formation (Kupfer et al., 2004; Roze et al., 2011; Strieker et al., 2010). Previous studies focusing on heterologous expression of biosynthetic pathways have, without exception, all utilized promoter reconstitution for activation of cluster specific transcription factors (Nielsen et al., 2013; Sakai et al., 2008; Yin et al., 2013) or all cluster genes (Fujii et al., 2016; Heneghan et al., 2010; Itoh et al., 2010) or for overexpression of a global

regulator like LaeA (Sakai et al., 2012). Alternatively, biosynthetic gene expression can be controlled using polycistronic expression cassettes separating genes with cleveage peptide signals (Hoefgen et al., 2018; Schuetze and Meyer, 2017), however such emerging methods are yet to be implemented for species of *Fusarium*. Fusing biosynthetic genes to alternative strong or inducible promoters serves multifold purpose; ensures expression and promotes, in theory, higher metabolite yield (Boecker et al., 2018; Kakule et al., 2015) which eases detection and metabolite isolation for functional or chemical analyses. In our experimental design we did not enhance the gene expression through genetic manipulation, as the *F. pseudograminearum* genes fused to their original promoters/terminators were functional in synthesizing the expected products in *F. graminearum*.

The crown rot causing *F. pseudograminearum* was originally regarded as a member of the *F. graminearum* speciation, but was later recoqnized as an individual species (Aoki and O'Donnell, 1999). The two species display separate morphological features and their genomes reveal a handful of noticeable differences: While they share 13 PKSs and 15 NRPSs, *F. graminearum* comprises *PKS1*, *PKS6*, *PKS52*, *NRPS7*, *NRPS8*, *NRPS17*, *NRPS18*, while *F. pseudograminearum* holds the *PKS40*, *NRPS32* and *FCK* genes (Brown and Proctor, 2016; Hansen et al., 2015; J. L. Sørensen et al., 2014). Lastly, *F. graminearum* causes head blight in cereals and form homothallic perithecia, while *F. pseudograminearum* is a heterothallic species (Aoki and O'Donnell, 1999). For activation of silent clusters in *F. graminearum*, controlling expression will be worth considering, especially for genes from less closely related species, because we do not know how phylogenetic distance effects the functionality of foreign genes (Rokas et al., 2018). It would be possible to develop a mixed method approach where promoter swapping is included in the vector assembly step. Alternatively, we can further modify and analyse gene clusters post introduction in the genome of *F. graminearum*, for instance applying recyclable markers allowing for several additional modifications (Connolly et al., 2018; Twaruschek et al., 2018). Finally,

expression of toxic metabolites can be controlled by inducible systems such as the tet-on promoters developed for *F. fujikuroi* (Janevska et al., 2017).

The pSHUT backbone is equipped with homology segments targeting integration towards the β-Tubulin locus. We chose this locus, as we have good experience expressing genes from this locus (Westphal et al., 2018b) and it resides in a euchromatic region of chromosome 4 (Connolly et al., 2013; Zhao et al., 2014). Meanwhile, we observed *Fg32-6* and *Fg32-8* transformants having potentially experienced several partial and ectopic cassette and backbone integration events. Unguided integration events may result in unintentional disruption of intrinsic genes, and result in unintended phenotypical changes compromising downstream experiments. However, in the context of using this system as a robust tool for production of biosynthetic compounds in *F. graminearum*, unintended sites and/or multi-copy insertion may be desirable and aid pathway characterization studies by providing higher production levels of intermediates or final cluster metabolites. Indeed the industrial strains of *Penicillium chrysogenum* used for penicillin production the producing cluster is present in multiple copies (Barredo et al., 1989; Smith et al., 1989).

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Tables

Table 1: yeast transformation reactions for SM cluster reconstruction in shuttle vectors

Gene cluster	Fungal DNA inserted (kb)	Shuttle vector	n PCR fragments
Fp Cytokinin (*FCK*)	9.944	pSHUT1 (2μ)	3
Fp NRPS32-PKS40 (*W493*)	54.471	pSHUT3-32 (CEN4/ARSH4)	16

Figure 1 Experimental work flow for cloning gene clusters and introducing gene clusters to the genomic DNA of *F. graminearum*. A. Structure of *F. pseudograminearum W493* gene cluster and primer positions for PCR amplification of 16 overlapping fragments (F1-F16) together comprising the full gene cluster (Supplementary Table T2). PCR fragments recombine with each other and the insertion site of linearized plasmid pSHUT3-32 through in vivo homologous recombination in *S. cerevisiae*. B. Structure of *F. pseudograminearum FCK* gene cluster and primer positions for PCR amplification (Supplementary Table T1). C. Resulting construct carrying an intact copy of the *W493* gene cluster. D. PCR validation of an isolated plasmid construct carrying the intact gene cluster; reactions validate recombination in correct order by utilizing primers producing bands spanning areas of homologous recombination (Supplementary Table T5). E. Intended targeted homologous recombination between linearized plasmid and integration locus of *F. graminearum*.

Figure 2 Biosynthetic pathway products in this study. A. Chemical structures of *FCK* pathway specific metabolites and intermediates. B. *W493* biosynthetic pathway products. C. *FCK* biosynthetic gene cluster. D. W*493* biosynthetic gene cluster. Shown in grey is homology to orthologous loci in *F. graminearum* PH-1 genome.

Figure 3 A. Mapping of *FCK*-cassette-associated reads to *F. graminearum* chromosomes 1, 2 and 3 and hypothetical chromosome 4 containing an inserted copy of the *FCK* gene cluster. B. Accumulated sequencing coverage of *FCK* gene cluster in β-Tubulin locus of mutant *FgCK-1* (forward reads; orange, reverse reads; blue). C. Chemical HPLC-HRMS analysis for *FCK* pathway metabolites in *FgCK-1* mutant and parental wild type strain *F. graminearum* PH-1. Extracted ion chromatograms are displayed as colored graphs for every mass.

Figure 4 Mapping *W493*-cassette-associated reads to *F. graminearum* chromosomes 1, 2 and 3 and hypothetical chromosome 4 containing an inserted copy of the *W493* gene cluster. A. Predicted cassette integration positions in transformant *Fg32-6*. B. Predicted cassette integration positions in transformant *Fg32-8*. C. Chemical HPLC-HRMS

analysis of *F. pseudograminearum*, *F. graminearum* parental and transformants *Fg32-6* and *Fg32-8*. Extraction ion chromatograms for $[M+H]^+$ masses: W493A (green) = 874.5284 ± 0.01 amu, W493B (orange) = 888.5440 ± 0.01 amu. D. Relative W493 A and B mass abundance in four fungi represented as an average from five technical replicates (only three replicates from *Fg32-8*). Error bars display standard deviation.

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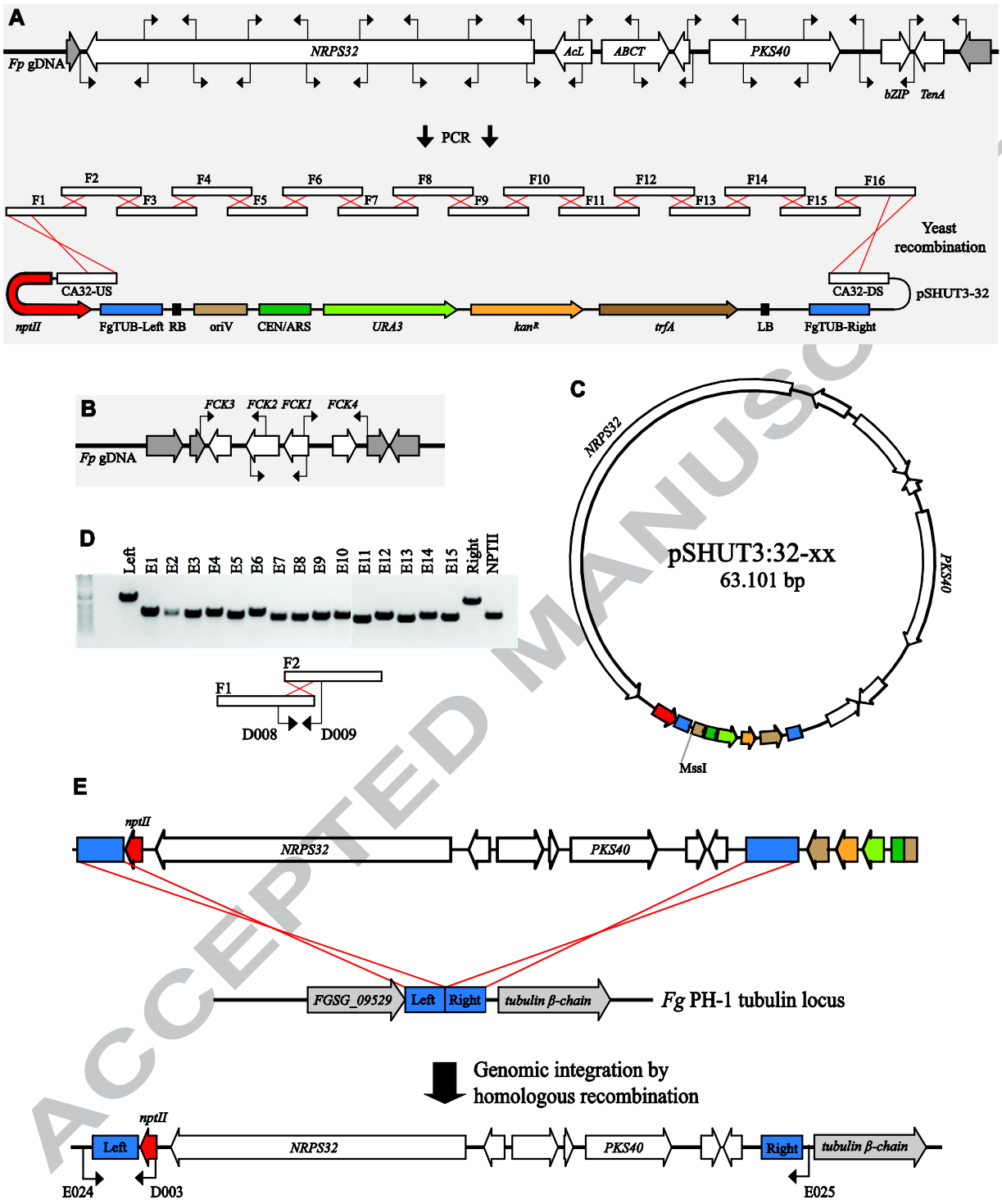

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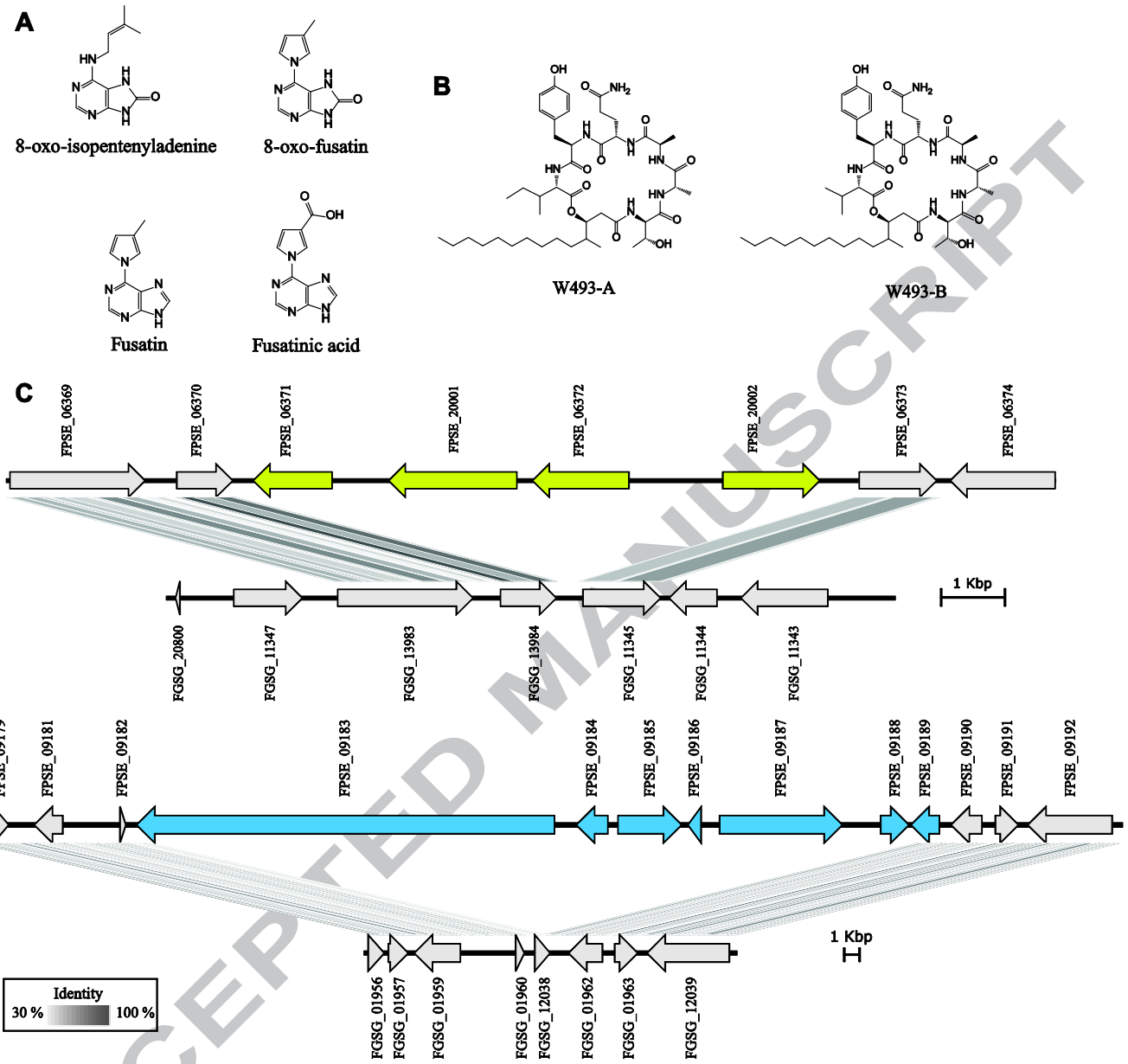

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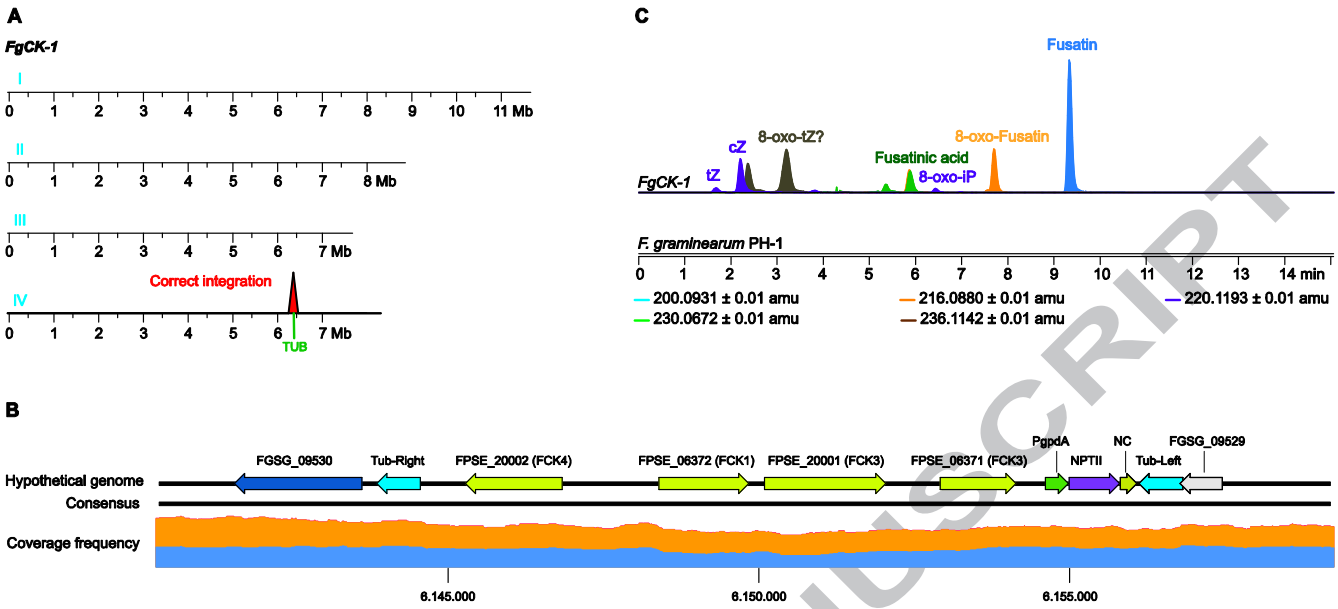

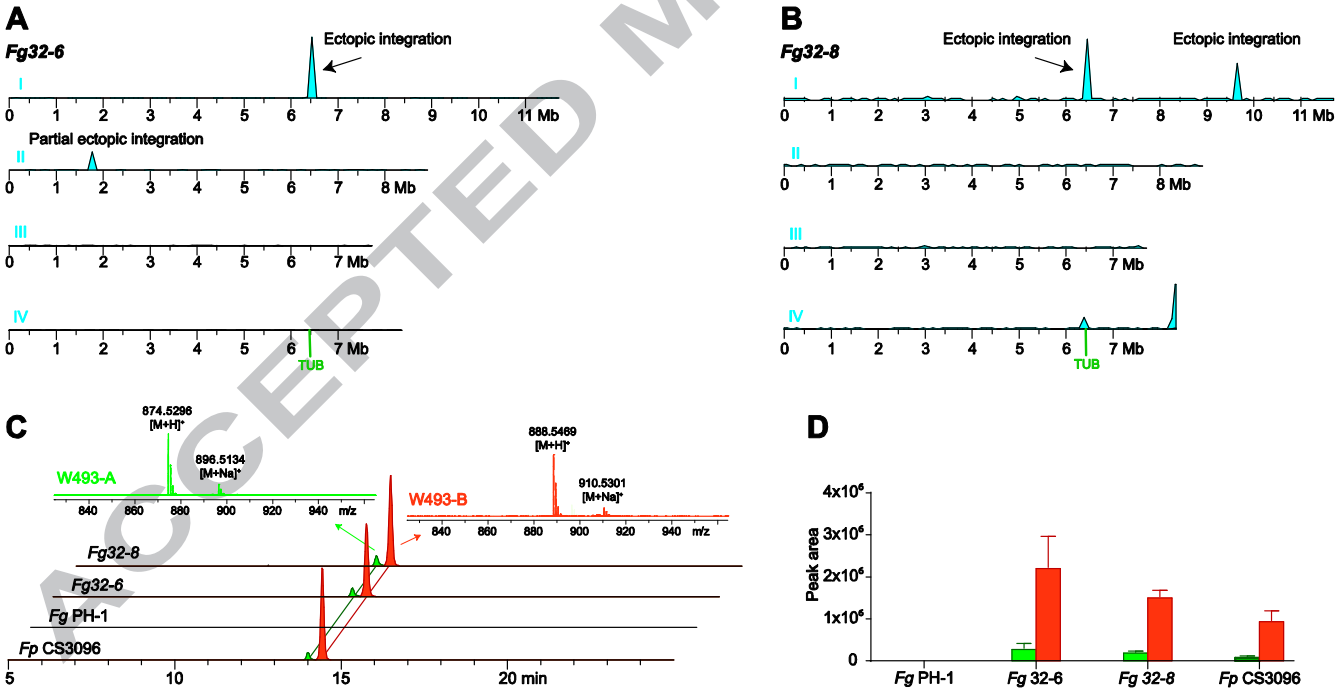

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Highlights

Heterologous production of the lipopeptide W493 and fungal cytokinins in *F. graminearum*

Yeast recombination enables reconstruction of large fungal gene clusters

Intact 10 and 54 kb foreign gene clusters were transformed via ATMT or PMT

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Graphical abstract