Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Functional analysis of polyketide synthases in the biocontrol fungus *Clonostachys rosea*

Umma Fatema

Forest Mycology and Plant Pathology
Master´s thesis • 45 hp hec
Genetics and Molecular Plant Science Master's program
Uppsala, Sweden 2017

Functional analysis of polyketide synthases in the biocontrol fungus *Clonostachys rosea*

Umma Fatema

Supervisor: Assistant Prof. Mukesh Dubey
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences (SLU)

Co-supervisor: Prof. Dan Funck Jensen
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences (SLU)

Associate Prof. Magnus Karlsson
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences

Examiner: Associate Prof. Malin Elfstrand
Department of Forest Mycology and Plant Pathology
Swedish University of Agricultural Sciences (SLU)

Credits: 45 hp
Level: Second cycle, A2E
Course title: Independent project in Biology- Master's thesis
Course code: EX0596
Programme/education: Genetics and Molecular Plant Science Master's program

Place of publication: Uppsala, Sweden
Year of publication: 2017
Part
Online publication: http://stud.epsilon.slu.se

Keywords: Biocontrol, Clonostahys rosea, Gene deletion, Polyketides, Polyketide synthase, Secondary metabolites

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Forest Mycology and Plant Pathology

Abstract

Clonostachys rosea, a filamentous ascomycete, is a well-known biological control agent (BCA) against a wide range of plant pathogenic fungi. Genome sequencing revealed that *C. rosea* strain IK726 possess 32 genes encoding for polyketide synthases (PKS), which is significantly higher than the more studied biocontrol fungi *Trichoderma.* During our previous study, we showed a culture medium dependent antagonistic effect of *C. rosea* against plant pathogenic fungi *Alternaria alternata*, *Botrytis cinerea*, *Fusarium graminearum*, and *Rhizoctonia solani*. Furthermore, we showed a positive correlation between *pks* gene expression and antagonism in *C. rosea*. The aim of the present study was to i) analyse the expression of *pks* genes in *C. rosea* under developmental stages and during *C. rosea* interaction with prey fungi; and ii) to characterize the biological function of one or few polyketide synthases in *C. rosea* by generating gene deletion mutants. The expression pattern of *pks* in *C. rosea* during pigmentation and during antagonistic interaction with plant pathogenic fungi *B. cinerea* and *F. graminearum* was investigated using quantitative polymerase chain reaction. Our results showed that 19 *pks* in *C. rosea* were significantly induced during pigmentation compared to the non-pigmented control. Our result also showed induced expression of 18 *pks* during *C. rosea* and *B. cinerea* interaction (Cr-Bc) compared to *C. rosea* self-interaction (Cr-Cr) or *C. rosea* and *F. graminearum* interaction (Cr-Fg), and 7 *pks* during Cr-Fg compared to Cr-Cr or Cr-Bc. Phenotypic analyses of *C. rosea* wild-type (WT), *pks22* and *pks29* gene deletion mutants (Δ*pks22* and Δ*pks29*) demonstrate that Δ*pks22* had a significant reduction ($P \leq 0.004$) in mycelial growth rate in comparison to WT. However, no significant difference in antagonism against *B. cinerea* or *F. graminearum* between WT and Δ*pks22* was found. On the other hand, deletion of *pks29* resulted in no significant difference in growth rate but reduction ($P \leq 0.011$) in antagonistic ability against *B. cinerea* or *F. graminearum* in *C. rosea*. These data show that the PKS is important for normal growth and development, and antagonism in *C. rosea*.

Table of contents

Abbreviations

ABC	ATP-binding cassette
ACP	Acyl carrier protein
ANOVA	Analysis of variance
AT	Acyltransferase
ATMT	*Agrobacterium tumefaciens*-mediated transformation
ATP	Adenosine try phosphate
BCA	Biological control agent
Bp	Base pair
C	Carbon
cDNA	Complementary DNA
CoA	Coenzyme A
Ct	Threshold cycle
CTAB	Hexadecyl-trimethyl-ammonium bromide
CZ	Czapek-dox
DAI	Days after inoculation
DH	Dehydratase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Enoyl reductase
Hyg	Hygromycin
IM	Induction medium
KR	Ketoreductase
KS	ketoacyl-CoA synthase
LB	Luria-Bertani
MFS	Major facilitator superfamily
N_2	Nitrogen
NaCl	Sodium chloride
NRPS	Non-ribosomal peptide synthetases
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PKS	Polyketide synthase
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction
SMART	Simple modular architecture research tool
SMS	Synthetic minimal salt
SNA	Synthetic nutrient agar
TE	Tris.-EDTA
UV	Ultraviolet

1. Introduction

1.1 Biological control

Plant diseases need to be controlled to maintain the quality and the quantity of foods and feeds for a growing human population. To avoid or mitigate plant disease farmers often rely on chemical fungicides. Overuse use of chemical fungicides has adverse effects on human health and environment (Horrigan *et al.*, 2002), and pose risk to fungicide resistance in pathogens (Hobbelen *et al.*, 2014). Biological control of plant diseases by biological control microorganisms is a promising alternative of chemical fungicides to ensure the safety of human health and environment while maintaining good quality and quantity of food and feed.

Biological control could be stated as 'any activity of one species that reduces the adverse effect of another' and the organism that suppresses the pest and pathogen are called biological control agent (BCA) (Pal & McSpadden Gardener, 2006). The biocontrol mechanism is attributed to a combination of successful i) competition for nutrients and space ii) antibiosis by secreting antibiotics and toxins iii) mycoparasitism by producing cell wall degrading enzymes, and iv) induction of resistance responses in host plant. A variety of bacterial and fungal BCAs has been commercialized against seed and soil borne diseases, and are available in the market viz- *Trichoderma* (Root shield, Supresivit, Binab, Trichodex, Tricho-Dry), *Gliocladium catenulatum* (is now called *Clonostachys rosea f. catelulata*) (GlioMix), *Conithyrium minitans* (ContansWG), *Agrobacterium (*NoGall), *Bacillus* sp. (Kodiak) *Pseudomonas* (Cedomon, Spot-Less), and *Streptomyces* (Mycostop). Beside the use of bacteria as a BCA, the growing interest to use fungi as BCA can be helpful to achieve sustainable crop protection strategy. Though the number of fungal BCAs in the market is growing, there is still a vast discrepancy between the academic research and practical applications. Many academically promising BCAs never reach to the practical usage. The hindrances are their inconsistency to control pathogens and lack of environmental adaptability. It is, therefore, important to understand the mechanisms of biological control of plant diseases. This will help to select effective BCAs and to maximize their use for sustainable crop production strategy.

1.2 Clonostachys rosea

The fungus *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Seifert & W. Gams, comb. nov., previously known as *Gliocladium roseum*, is an ascomycete in the Bionectriaceae family and

is reported as the anamorph of the teleomorph *Bionectria ochroleuca* (Schw.) Shroers & Samuel (Schroers *et al.*, 1999). *C. rosea* has a wide geographical distribution from subarctic to the tropical region and is also stated as a common saprophyte in soil (Toledo *et al.*, 2006; Zhai *et al.*, 2016; Wang *et al.*, 2017). *C. rosea* is characterized by unpigmented aerial mycelium and dimorphic conidiophores (primary conidiophores and secondary conidiophores) (Schroers *et al.*, 1999). The verticillium-like primary conidiophores arise first and penicillate-like secondary conidiophores arise later. The shape of conidia produced from each type is also different (Schroers *et al.*, 1999). Conidia from primary conidiophores are less curved and larger than the conidia from secondary conidiophores.

The antagonistic/mycoparasitic activity of fungus *C. rosea* has been shown against numerous plant pathogenic fungi for example *Botrytis cinerea* to control grey mold (Mouekouba *et al.*, 2014), *Fusarium oxysporum* to control *Fusarium* root and stem rot (Chatterton *et al.*, 2008), *Plasmodiophora brassicae* to control clubroot (Lahlali & Peng, 2014), and *Sclerotinia sclerotiorum* (Sun *et al.*, 2015). Moreover, *C. rosea* acts as a potential parasite against nematodes (Ahmed *et al.*, 2014). In addition, *C. rosea* can colonize the plant roots which results in plant growth promotion and induction of plant defence response (Ravnskov *et al.*, 2006; Roberti *et al.*, 2008; Chatterton & Punja, 2010).

In recent years, *C. rosea* is attaining interest by the researches as an important BCA because of its mycoparasitic ability to parasitize and kill other fungi (Krauss *et al.*, 2013). But the modes of action of *C. rosea* as BCA are not fully understood though antibiosis, enzymatic activity, and induced resistance are reported to play important roles (Roberti *et al.*, 2008; Mamarabadi *et al.*, 2009; Mouekouba *et al.*, 2013; Lahlali & Peng, 2014; Mouekouba *et al.*, 2014) and the mechanisms of their biocontrol activity vary among the strains of *C. rosea* (Xue, 2003; Jensen *et al.*, 2007; Hue *et al.*, 2009).

1.3 *C. rosea* strain IK726 as a biological control agent

C. rosea strain IK726 was originally isolated from barley roots infected with *F. culmorum* in Denmark (Knudsen *et al.*, 1997). *C. rosea* IK726 has been reported as an efficient BCA against seed and soil borne pathogenic fungi under field conditions (Knudsen *et al.*, 1995, 1997; M⊘ller *et al.*, 2003; Hue *et al.*, 2009). As for example, in the study of Jensen *et al.* (2004), bio-priming of carrot seeds with *C. rosea* IK726 reduced the incidence of *Alternaria radicina* from 29% to <2.3% and incidence of *A. dauci* from 11% to <4.8%. In the same study,

the higher number of healthy seedling stands was observed in bio-primed seeds compared with hydro-primed (treatment with water) and non-primed (control) seeds. Furthermore, *C. rosea* IK726 has been reported to have general stimulating effects on soil enzyme activity and the soil microbiota (Johansen, 2005; Ravnskov *et al.*, 2006), which ultimately assist in plant growth promotion with the subsequent increase in plant dry weight. The most advanced mechanism to suppress plant pathogen by the activity of BCA-produced enzymes such as chitinases and glucanases has also been proved in *C. rosea* (Mamarabadi, 2008). Moreover, this strain has ability to tolerate *Fusarium* spp. mycotoxin zearalenone (ZEA), and the fungicides Cantus, Chipco Green and Apron by mediating active efflux of toxic metabolites across the cellular membrane with the help of ATP-binding cassette (ABC) transporters (Dubey *et al.*, 2014a).

The genome of *C. rosea* IK726 has been sequenced (Karlsson et al., 2015). Analysis of gene family evolution showed a significant expansion of gene family known to involved in pectin degrading activity (pectin lyase), synthesis of secondary metabolites (polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS)), and membrane transportation (ATP-binding cassette (ABC) transporters) in *C. rosea* in comparison to other biocontrol fungi such as *T. atroviride* and *T. virens* and saprophytes *T. reesei* along with other closely related hypocreals (Karlsson et al., 2015). Functional analysis of genes of these families will help to understand the life strategies and biocontrol mechanisms in *C. rosea*. Recently, functional analysis of gene encoding ABC transporters showed a role of membrane transporters in mycoparasitic activity and xenobiotic tolerance in *C. rosea* (Dubey *et al.*, 2014a, 2016), but the role of secondary metabolites especially PKS during mycoparasitism is yet to be investigated.

1.4 Polyketides and polyketide synthases

Polyketides are a group of secondary metabolites, naturally produced by both prokaryotes (bacteria) and eukaryotes (fungi, algae, higher plants, and animals). They are synthesized from several acyl units by polyketide synthases (PKSs; encoded by PKS genes). PKS catalyse successive condensation reactions of acyl-CoAs to produce polyketomethylene intermediates and some subsequent reactions such as cyclization and reduction of the intermediates (Watanabe & Ebizuka, 2004). PKSs are generally categorized into three types viz- Type I, Type II and Type III PKS. Type I PKSs are further divided as iterative and non-iterative type. The iterative type contains all the necessary domains in a single module and their active sites are used repeatedly to produce a single polyketide. On the other hand, the non-iterative type

contains more than one module to constitute specific domain and the active sites are used only once to produce a particular polyketide (Timsina, 2012). Type I and type II PKSs are found in bacteria and fungi. Bacteria possess both iterative and non-iterative type I PKS whereas fungi possess mainly iterative type. Type III PKSs are found in bacteria, fungi, and higher plants. Type III fungal PKSs are less abundant than Type I fungal PKS and the importance of type III fungal PKSs is not well known (Hashimoto *et al.*, 2014). Among the polyketides in all kingdoms, fungal polyketides are most abundant and diverse. The diversity of fungal polyketides depends on number and modes of iterative reactions, reduction reactions, and cyclization (Keller et al., 2005) during synthesis of polyketides.

The synthesis mechanism and domain structure of fungal Type I PKS are very similar to eukaryotic fatty acid synthesis. Among the 11 different catalytic domains of PKS, the ketoacyl-CoA synthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains are essential for both fungal polyketides and fatty-acid synthesis, whereas the ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains, required for ketone reduction in fatty acid synthesis may either present (reducing PKS) or absent (non-reducing PKS) in fungal PKS (Keller *et al.*, 2005; Timsina, 2012).

1.5 The function of fungal polyketides

To date, numerous functions have been described for fungal polyketides. Most of the fungal polyketides function for ecological and evolutionary adaptation of fungi (Fujii, 2010). They serve as precursors to toxins in fungi which have adverse impacts on human, animal and plant health. As for example, aflatoxin from *Aspergillus flavus* causes yield reduction in agricultural commodities and responsible for carcinogenic effects in animal (Fakruddin *et al.*, 2015), aurofusarin from *F. graminearum* affects the nutritional quality of quail egg (Dvorska *et al.*, 2001), fumonisisn from *F. moniliforme* causes human esophageal cancer (Gelderblom *et al.*, 1988), and zearalenone from several species of *Fusarium* implicates reproductive disorders of farm animals and hyper oestrogenic syndromes in humans (Zinedine *et al.*, 2007). Polyketides also function for pigmentation of fruiting body and spores in fungi. The best-studied examples include the blackish perithecial pigment 'melanin' in *Sordaria macrospora*. (Engh *et al.*, 2007), dark perithecial pigment 'fusarubins' in *F. fujikuroi* (Studt *et al.*, 2012), red perithecial pigment in *Nectria haematococca* (anamorph *F. solani*) (Graziani *et al.*, 2004), and red ascospore pigment 'ascoquinone A' in *A. nidulans* (Brown & Salvo, 1994). Pigment-forming PKS function for virulence and abiotic stress resistance in fungi beyond providing the colour of

perithecia and conidia (Howard *et al.*, 1991; Beltrán-García *et al.*, 2014). In *Magnaporthe grisea*, for example, only dark melanised appressorium with high turgor pressure were reported to penetrate the plant cells (Howard *et al.*, 1991), and in banana pathogen *Mycosphaerella fijiensis*, light activated DHN-melanin acts as a virulence factor by producing reactive oxygen species (ROS), responsible for the cellular death of the pathogen (Beltrán-García *et al.*, 2014). The abiotic stress resistance in fungi by pigment forming PKS has also been revealed in several studies. For example, the pigment layer of spores increased their resistance capacity to UV radiation in *Trichoderma* (Atanasova *et al.*, 2013). PKS are also linked to growth and sexual development, conidiation and survival capability of ascomycete fungi. A significant decrease in mycelial growth was reported in *pks* disruption mutants in *Gibberella zeae* (anamorph *F. graminearum*) (Gaffoor *et al.*, 2005). In another filamentous ascomycete *S. macrospora*, deletion and overexpression of *pks4* revealed its necessity to regulate sexual development and fruiting body morphology (Schindler & Nowrousian, 2014). Many polyketides are commercially important because of their ability for producing, antifungal, anticancer, anti-tumour and cholesterol lowering compound (Weissman & Leadlay, 2005; Campbell & Vederas, 2010; Bladt *et al.*, 2013).

To date, only few polyketides has been reported for facilitating the mycoparasitic ability of potential BCAs against pathogenic fungi. For example, in the biocontrol fungus *T. reesei*, loss of *pks4* resulted in reduced antagonistic potential against pathogenic fungi *A. alternata*, *R. solani*, *S. sclerotiorum* (Atanasova *et al.*, 2013). In another study, differential regulation of *pksT-1* and *pksT-2* in *T. harzianum* 88 during plate confrontation assay with *F. oxysporum*, *R. solani*, or *S. sclerotiorum* revealed their regulatory roles as mycoparasitic compounds (Yao *et al.*, 2016).

1.6 PKS in *C. rosea* IK726

Genome sequence analysis of *C. rosea* IK726 has shown that *C. rosea* possesses 32 PKS encoding genes (Karlsson *et al.*, 2015). This number of PKS in *C. rosea* is twice as high compared with the plant pathogen *Fusarium* (16 *pks*, highest *pks* among the *Fusarium* species), and more than twice as high as mycoparasitic *Trichoderma* (18 pks, highest number of *pks* among the *Trichoderma* species) (Karlsson *et al.*, 2015). The PKS of *C. rosea* have been categorized into reducing and non-reducing type based on the alignment of keto synthase domains of PKS along with the functionally characterized *pks* of other ascomycetes and bacteria (Figure 1.) (Karlsson *et al.*, 2015). Among the 32 PKS encoding genes in *C. rosea*, 4

PKS genes (*pks1-pks4*) code for non-reducing, and 25 PKS genes (*pks5-pks29*) code for the highly reducing type. The sequences of *pks30* and *pks31* are incomplete, and *pks32* is a hybrid of PKS and NRPS.

However, to date, none of the PKSs in *C. rosea* IK726 has been characterized. To reveal their regulatory and functional behaviour, during my master's program, we first analysed *pks* gene expression in *C. rosea* under different nutritional conditions. Gene expression analysis showed that the *pks* expression is nutrition dependent. In addition, our work showed a positive correlation between *pks* gene expression, and antagonism against phytopathogenic fungi *A. alternata, B. cinerea, F. graminearum,* and *R. solani* in different growth medium (Umma Fatema, research training project; 15 hp).

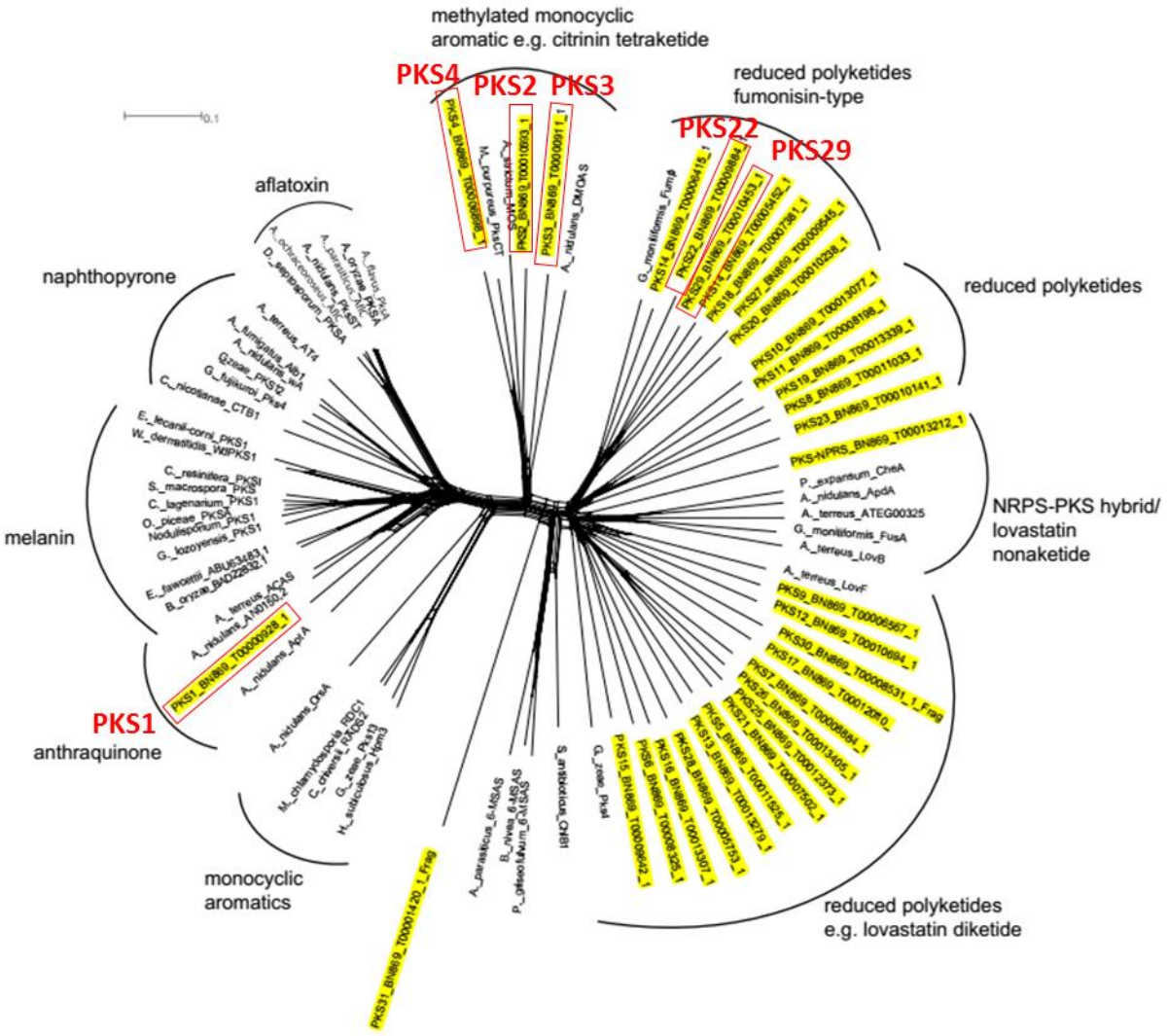


Figure 1. Phylogenetic network of *pks* of *C. rosea* along with functionally characterized *pks* of ascomycetes and bacteria. This figure is obtained from the study of Karlsson *et al*., (2015). The *pks* of *C. rosea* are highlighted in yellow. The red rectangular marked PKS encoding genes (PKS1, PKS2, PKS3, PKS4, PKS22, and PKS29) were deleted from *C. rosea* genome in this study to generate deletion mutants.

2 Aim and objectives of the study

The overall aim of this study was to investigate the biological function of PKS to advance the current understanding of the biological control mechanisms in the fungus *C. rosea*. The study was undertaken with two main objectives:

a. Expression analysis of genes encoding polyketide synthase in *C. rosea* under developmental stages and during interaction with prey fungi.
b. Functional characterization of the selected polyketide synthases by generating gene deletion mutants.

3 Materials and Methods

3.1 Fungal strain and culture conditions

Fresh culture of *C. rosea* IK726, *F. graminearum,* and *B. cinarea,* obtained from Department of Forest Mycology and Plant Pathology were maintained on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO) plates at 25°C in the dark.

3.2 Sequence analysis

The genome of *C. rosea* IK726 has already been sequenced and published (Karlsson *et al.*, 2015). The nucleotide sequences of 32 PKS encoding genes were provided by Mukesh Dubey, Department of Forest Mycology and Plant Pathology. The domain architectures of 32 PKS of *C. rosea* IK726 were analysed and annotated based on their amino acid sequences using simple modular architecture research tool (SMART) protein analysis tool (Schultz *et al.*, 1998).

3.3 Primer designing

All the primers, used in this study were designed using PrimerSelect (DNASTER Lasergene 11 core suite) following the software guidelines (Premier Biosoft). Nucleotide sequences of respective *pks* of *C. rosea* were used for primer designing

3.4 Gene expression analysis

Gene expression analysis of 32 PKSs in *C. rosea* was carried out in 2 different conditions: i) during pigmentation of *C. rosea* and ii) during interactions of *C. rosea* with plant pathogenic fungus *F. graminearum* or *B. cinerea.* For gene expression analysis during pigmentation, a 5 mm agar plug of *C. rosea* was inoculated on PDA medium covered with cellophane membrane, and incubated at 25 °C in dark for 10 days. The *C. rosea* agar plug was cultured on the membrane for easy harvesting of fungal mycelia. The culture plates were wrapped with aluminium foil to ensure their growth in the dark to avoid conidiation. Four days old *C. rosea* culture plates, incubated at the same conditions was used as control treatment. Harvested mycelia were flash frozen in liquid nitrogen, freeze-dried (VirTis Sp scientific, Warminster, PA) at -95 °C for three days, and homogenized into the fine powder using Precellys 24 lysis and homogenization (Bertin Technologies, France) device at 5000 rpm for 28 second (two times with 5 second pause). Immediately after homogenization, total RNA extraction from all the samples was done using Qiagen RNasy plant mini kit (Bio-rad, Hilden, Germany) following

manufacturer's protocol. For gene expression analysis during interactions of *C. rosea* with *F. graminearum* (Cr-Fg) or *B. cinerea* (Cr-Bc), total RNA of *C. rosea* from the confronted plates was provided by Mukesh Dubey, Department of Forest Mycology and Plant Pathology.

Total RNA from all the samples was treated with DNase I (Fermentas, St-Leon-Rot, Germany) following manufacturer's instruction and concentration was determined with the Nanodrop (ND-1000 Nanodrop spectrophotometer, Seven Werner). Complementary DNA (cDNA) was synthesized using 1000 ng of total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's descriptions. Gene expression analysis was performed by real-time quantitative polymerase chain reaction (RT-qPCR) using *pks* specific primer pairs (Table 1) in a 20 µl reaction mix with 10 µl of 2 × SsoFast EvaGreen Super mix (Bio-Rad, Hercules, CA, USA.), 0.3 µl each of forward and reverse primer (10 pmol/µl), 5 µl of *C. rosea* cDNA, and 4.4 µl of milli-Q water. Initial denaturation was carried out at 98 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 5 sec, annealing at 60 °C for 10 sec, and the final extension at 65 °C for 10 sec in an iQ5 qPCR system (Bio-Rad, Hercules, CA, USA). Primer amplification efficiency was determined by amplification of serial dilutions of *C. rosea* IK726 genomic DNA using PCR conditions described above. Relative expression of *pks* was analysed from threshold cycle (Ct) values using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and relative expression of β-tubulin was used for standardisation of *pks* relative expression data (Dubey *et al.*, 2014a). Thereafter, a series of sequential corrections including log transformation, mean centering, auto-scaling were applied to standardize variation in gene expression data among independent biological replicates as described previously (Willems *et al.*, 2008). The gene expression experiments were performed with five biological and two technical replicates.

Table 1: RT-qPCR primers used in this study for target gene amplification

Primer name	Sequence 5´-3´	Sequence 3´-5´	Target	Amplicon size (bp)
PKS1	tggggttttactgggtgtgctc	gatacccagcccagtaaccacag	*pks1*	159
PKS2	tctcggctactggtgctctaagg	ttggaagaacatgggcgtagtg	*pks2*	205
PKS3	gtggtgggggaacttttgaac	gcagccataatctaggggttcag	*pks3*	183
PKS4	gctgcacctttccactcgttc	gtaaactgctgctgaaacggactc	*pks4*	129
PKS5	ctccggcctgctaaagttgttg	gtaagccgagtgccatcatagagc	*pks5*	186
PKS6	tccccaacgaacgcatctttag	cgttcaactgagcgtccttctttc	*pks6*	188
PKS7	cattcggggcacagacacaaac	gcttcgatgtagcgtgtttggtc	*pks7*	137
PKS8	caccgcatgttcatcctcactc	acgcccattctcgctgtattc	*pks8*	140
PKS9	aataaaccacggcccttccttc	accatcccacttgctatccgtc	*pks9*	197
PKS10	acatctcccgtccgtttcagtg	gcaaggctcagcagggtaacag	*pks10*	187
PKS11	catgggggattcaagcagagtc	gacgcatgcaacacaaacacg	*pks11*	175
PK12	caaatggcaggggagtagacg	gaaactgagcgggagaaggaac	*pks12*	162
PKS13	atggggaaggctatgggctatg	ctattcaaggccgccaccatc	*pks13*	202
PKS14	gaggctggctcgaatcaagac	tgcatagtcaccagcgtccag	*pks14*	192
PKS15	gttgcagggctgatcaaaggag	ggccagtccattaatcgtgtagg	*pks15*	136
PKS16	aggcagcagtcattcctatcgtg	gcttctcaagactgccgactgtg	*pks16*	200
PKS17	gaatgccatggaacaggaacac	gcctacgcttggtttcacactg	*pks17*	131
PKS18	cgttgatgcgttaagcgagattg	ccatggctccagctttctttacc	*pks18*	169
PKS19	gcaatgaaccaacagcgaacc	cctcttgtcggtgggataatgc	*pks19*	151
PKS20	gttccccttttgcctgctacattg	gatgctgaactcccaccactgac	*pks20*	201
PKS21	tctgcccaagctcctcgtattc	gatctccataacatgcggctgc	*pks21*	164
PKS22	tggaaagagctgcagacgagag	gagcgacaagggctgatgaac	*pks22*	156
PKS23	gatgtctttgtcgccagcctc	ctccgagacatccgtaaaggtg	*pks23*	191
PKS24	aaacccgcttcatcaacgacac	cgaggcgtcaaactgggataga	*pks24*	124
PKS25	gccaatgatgatgcggtgaac	caaattcagcgggtccatctc	*pks25*	200
PKS26	gcactggtacgcagattggagac	gatggccgatgtttgctttgatag	*pks26*	119
PKS27	tgcacgcagcaggtctcaac	gcagaactcatcgggcacaac	*pks27*	202
PKS28	gagacgctccatccaaacatcc	tggtcgccgttctcaatcactac	*pks28*	151
PKS29	tggtgggaattcagcatctcg	gaatcgcggtccgtagttttg	*pks29*	197
PKS30	ggtatgaaaagcccgcaatcc	ggctgtgtactttgggatgacg	*pks30*	182
PKS31	catcatgatgggggctacagg	cagcgtcgcctaaagcaatg	*pks31*	186
PKS32	atccttcgagcgtggggtatc	acccttcctgctgggtcattc	*pks32*	195

3.5 Construction of deletion cassettes

The deletion cassettes were generated according to the protocol of Gateway® technology (Invitrogen, Carlsbad, CA, USA). The specific attB sequences were added at 5' end of PCR primers to allow cloning of the amplified products into specific Gateway® donor vectors to

generate entry clone from upstream and downstream region of gene of interest in a BP recombination reaction. Thereafter, entry clones are ligated and cloned into destination vectors. The specific attB sequences and their specific adjoining sites are given:

	Sequence 5´-3´	**Specific adjoining site**
attB4	ggggacaactttgtatagaaaagttg	upstream forward primer of *pks*
attB1r	ggggactgctttttgtacaaacttg	upstream reverse primer of *pks*
attB2r	ggggacagctttcttgtacaaagtgg	downstream forward primer of *pks*
attB3	ggggacaactttgtataataaagttg	downstream reverse primer of *pks*

3.5.1 Gene amplification and purification

Genomic DNA of *C. rosea* was isolated following a Hexadecyl-trimethyl-ammonium bromide (CTAB)-based protocol available in the department of Forest Mycology and plant pathology. Genomic DNA was used to amplify 5´ and 3´ flanking regions of *pks1, pks2, pks3, pks4, pks22,* and *pks29* using primer pairs PKS1ups F/ PKS1ups R and PKS1ds F/ PKS1ds R; PKS2ups F/ PKS2ups R and PKS2ds F/ PKS2ds R; PKS3ups F/ PKS3ups R and PKS3ds F/ PKS3ds R; PKS4ups F/ PKS4ups R and PKS4ds F/ PKS4ds R; PKS22ups F/ PKS22ups R and PKS22ds F/ PKS22ds R; PKS29ups F/ PKS29ups R and PKS29ds F/ PKS29ds R respectively. List of primers used for target product amplification is provided in Table 2. The PCR was carried out in a 25µl reaction with 0.3µl of Dream-Taq polymerase, 1 µl of DNA (50 ng/µl), 2.5 µl of Dream-Taq green buffer, 2.5 µl of dNTPs, and 0.375 µl each of forward and reverse primer (10 pmol/µl). In the PCR reaction, initial denaturation was at 95 ºC for 3 min followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min, which was followed by final extension at 72 °C for 10 min. Amplified products were analysed by agarose gel electrophoresis, and were purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA).

3.5.2 Gateway cloning

The BP recombination reactions were carried out according to the manufacturer's instruction of Gateway® technology (Invitrogen, Carlsbad, CA, USA). To generate entry clone from 5´ flanking region donor vector pDONR P4-P1r (Invitrogen, Carlsbad, CA, USA) and entry clone from 3´ flanking region donor vector pDONR P2r-P3 (Invitrogen, Carlsbad, CA, USA) were

used. The BP recombination reactions were then transformed into One Shot® TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's procedure, and were selected on the LB-agar (Luria-Bertani; 10g/L bactotryptone, 10 g/L yeast extract, 10 g/L NaCl, 15 g/L bactoagar) plate containing 50 µg/ml of selection antibiotic Kanamycin (Sigma-Aldrich, St. Louis, MO). Plasmids were isolated using QIAGEN Plasmid Isolation Kit (QIAGEN, Germantown, MD, USA) from overnight grown culture. Restriction analysis was performed on isolated plasmids to confirm correct insertions of the fragments. Restriction enzymes were selected based on vector map provided in supplementary Figure S1. Correct clones were proceeded to LR recombination reaction. The gateway entry clone for hygromycin resistance gene cassette (*hygB*) was offered by Mukesh Dubey, Department of Forest Mycology and Plant Pathology.

Using the LR recombination reaction, the entry clones of 5′ flanking regions, 3′ flanking regions of target *pks*, and *hygB* were ligated together into destination vector pPm43GW (Karimi *et al.*, 2005) to create the deletion vector following manufacturer's descriptions (Invitrogen, Carlsbad, CA, USA). The LR reaction mix was transformed into One Shot® TOP10 *E. coli* competent cells (Thermo Fisher Scientific, Waltham, MA, USA), and selected on spectinomycin (100 µg/ml; Sigma-Aldrich, St. Louis, MO) containing LB-agar plate. Plasmids from overnight grown culture were isolated maintaining the steps mentioned in BP recombination reaction. Restriction analysis of isolated plasmids was carried out to confirm the correct insertion.

3.6 Agrobacterium mediated transformation

The deletion vector was transferred into *Agrobacterium tumefaciens* strain AGL-1 according to freeze-thaw procedure (Xu & Li, 2008), and positive colonies were selected on LB-agar plates containing 50 µg/ml carbenicillin (Sigma-Aldrich, St. Louis, MO), 50 µg/ml rifampicin (Sigma-Aldrich, St. Louis, MO), and 100 µg/ml spectinomycin (Sigma-Aldrich, St. Louis, MO). Plasmids isolated from AGL1 cells were used for PCR using primers specific to hygromycin resistance gene *hph* and upstream or downstream region of respective *pks* to select the correct transformants (Table 2). *A. tumefaciens*-mediated transformation (ATMT) of *C. rosea* was performed following a protocol described in Utermark & Karlovsky (2008). In brief, *A. tumefaciens* cells harbouring deletion vector was grown in LB till OD_{600} reached to 0.5-0.9. The *A. tumefaciens* cells were harvested and allowed to grow overnight in induction medium (IM) containing 200 µM acetosyringone (Sigma-Aldrich, St. Louis, MO) to pre-induce the T-

DNA machinery. Thereafter, a mixture of *C. rosea* conidial suspension (1×10^7) and pre-induced *A. tumefaciens* cells was prepared in 1:1 ratio, and spread uniformly on to the surface of sterilised cellophane membrane, placed on solid IM agar plates containing 200µM acetosyringone. The plates were co-cultivated at 23 °C in darkness for 57 hours and cellophane membrane with dense layer of *C. rosea* mycelium was then transferred onto freshly prepared GM7 plates containing 300 µM of cefotaxime (Sigma-Aldrich, St. Louis, MO) to inhibit the growth of *A. tumefaciens* and 200 µg/mL of hygromycin B (Sigma-Aldrich, St. Louis, MO) as selection agent for fungal transformants. After 5-7 days of incubation, growing colonies were sub-cultured to freshly prepared selection plates containing the same concentration of hygromycin B.

Table 2: Primers for the validation of transformants

Primer name		Sequence 5´-3´	Target amplification
PKS1ups	**F**	attB4GCTTTCCGTGCATGGCTACAA	upstream region of *pks1* for deletion construct
	R	attB1rAAGAGGAAATGGCCCAGTATGCT	
PKS1ds	**F**	attB2rCTCGCTCAGAACAATCGACATAAG	downstream region of *pks1* for deletion construct
	R	attB3AAACTACCTAGAAGCCAAAGACGG	
PKS1ko	**F**	GATGAGGCGGAATGGGTAAATG	100-200 bp upstream (forward primer) and downstream (reverse primer) to *pks1* deletion cassette
	R	TTCTGTTAGCGAGACCAATCCCT	
PKS2ups	**F**	attB4CTTGAGCGCCGATAGACTGTAAC	upstream region of *pks2* for deletion construct
	R	attB1rCACCAACGACAGCAGGAACAT	
PKS2ds	**F**	attB2rAGGACAAAATAACGATGGCTAACC	downstream region of *pks2* for deletion construct
	R	attB3TTCTCAGTATTTGTTGCCTCTCCA	
PKS2ko	**F**	ATCCGATTTATGCACTTGTTGG	100-200 bp upstream (forward primer) and downstream (reverse primer) to *pks2* deletion cassette
	R	GCATCGAATAGCCCAACATCTT	
PKS3ups	**F**	attB4TACAGATGAGGTGGCACTTGGAG	upstream region of *pks3* for deletion construct
	R	attB1rATAAAGGTTCAAAAGCCCGAGAG	
PKS3ds	**F**	attB2rTGCTCGACCCAACAAAACAATA	downstream region of *pks3* for deletion construct
	R	attB3GATCCATCCAGTCCTTCCTCCT	
PKS3ko	**F**	ACGAGCTGCATTTCCCTAACA	100-200 bp upstream (forward primer) and downstream (reverse primer) to *pks3* deletion cassette
	R	CCTCGGGCTTCGTCTTATTCTT	
PKS4ups	**F**	attB4GTCTTATGGGGATGGTTTCACAC	

	R	attB1rGAAATTACGGTAGCTCCCTGTCC	upstream region of *pks4* for deletion construct
PKS4ds	**F**	attB2rATATGGAATGCCGAAGCGAGA	downstream region of *pks4* for deletion construct
	R	attB3TAGGGCATCAGGGGTTAGGGT	
PKS4ko	**F**	CACACCCGCATCAGAAATAACA	100-200 bp upstream (forward primer) and downstream (reverse primer) to *pks4* deletion cassette
	R	AGCCAATTTGACACCACCTAAGAC	
PKS22ups	**F**	attB4GACCCGCACCAACTTGAGACTAT	upstream region of *pks22* for deletion construct
	R	attB1rCCCTACCCACAATCAACCAACTT	
PKS22ds	**F**	attB2rCTTCCACAGGAGAGGGGACTTAC	downstream region of *pks22* for deletion construct
	R	attB3ACAATCAGATCCACCAGCCTACC	
PKS22ko	**F**	ACTTTGAGCCTGGCATCGTATC	100-200 bp upstream (forward primer) and downstream (reverse primer) to *pks22* deletion cassette
	R	TGAGGTGATTGGGAGCGTAGAT	
PKS29ups	**F**	attB4TGTGTCCATGAGGTGTGAAGTCC	upstream region of *pks29* for deletion construct
	R	attB1rCGTCTCGGTTTCTGTGTTGTTTTG	
PKS29ds	**F**	attB2rGCGAGTTGAAGATGACGAAGGAT	downstream region of *pks29* for deletion construct
	R	attB3ATAACCGGATGCAAAACAGTCTTC	
PKS29ko	**F**	GGAACTCGGCTTCTTACAACTGAC	100-200 bp upstream (forward primer) and downstream (reverse primer) to *pks29* deletion cassette
PKS29ko	**R**	CTTTGGAACCACTGCCTTGACTT	
Hyg	**F**	GCGCGCAATTAACCCTCAC	Hygromycin (*hygB*) cassette
	R	GAATTGCGCGTACAGAACTCC	

3.7 Validation of mutants

DNA was extracted from the sub-cultured transformants using CTAB method. A PCR approach was used to confirm the homologous insertion of deletion cassette using primer pair specific to *hygB* and sequences flanking 100-200 bp upstream region or downstream region of the deletion cassette. The PCR was carried out using DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with conditions: initial denaturation at 95 ^{o}C for 3 min followed by 32 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 20 sec, and extension at 72 °C for 3 min, followed by final extension at 72 °C for 10 min. Furthermore, reverse-transcriptase PCR (RT-PCR) on cDNA synthesized from RNA isolated from the PCR positive mutants along with WT was carried out using gene specific primers. Single conidium

was purified from the verified transformed mutants and cultured on PDA medium for phenotypic analysis.

3.8 Phenotypic analysis

3.8.1 Analysis of morphology, growth rate and conidiation

Two different culture media were used for phenotypic analysis. a) Czapek-dox (CZ; Sigma-Aldrich, St. Louis, MO); (in g/L) sucrose, 30, NaNO3, 2, K2HPO4, 1, MgSO4, 0.5, KCL, 0.5, FeSO4, 0.01, pH 7.3 ± 0.2 and b) Potato dextrose broth (PDB; Sigma-Aldrich, St. Louis, MO); (in g/L) 24 (potato starch 4 , dextrose 20), pH 5.1 ± 0.2. A 3mm agar plug from *C. rosea* strains was inoculated onto PDA or solid CZ medium in a 5 cm petri plate and cultured at 25 °C. Colony morphology was recorded daily and colony diameter was measured on 5 days after inoculation (DAI) to determine the growth rate of the growing colony. Conidia were harvested from WT, and deletion strains 12 DAI in 10 ml distilled water and filtered using Miracloth (Sigma-Aldrich, St. Louis, MO). The concentration of the harvested conidia was determined under the microscope using a bright line haemocytometer according to the manufacturer's instruction (Sigma-Aldrich, St. Louis, MO). The experiments were performed in four biological replicates.

3.8.2 Antagonism test

An in vitro plate confrontation assay on CZ or PDA medium was performed to test the antagonistic behaviour of WT and deletion strains against pathogenic fungi *A. altarnata*, *B. cinerea* or *F. graminearum*. A 3 mm agar plug of *C. rosea* mutants or WT was inoculated at the edge of a 9 cm agar plate and incubated at 25 °C for 7 days. A 3 mm agar plug of *A. altarnata*, *B. cinerea* or *F. graminearum* was inoculated on opposite edge of the plate and incubated at 25 °C. Linear growth of both *C. rosea* and pathogenic fungi was recorded daily. Lytic zone in between *C. rosea* and *F. graminearum* mycelium was measured 10 DAI.

For secretion assay, fresh *C. rosea* conidia (1×10^7 conidia/ml) of deletion or WT strains were incubated for 6 days in 200 ml CZ broth or PDB medium at 25 °C and 100 rpm on a rotary shaker. Culture filtrates were obtained by removing fungal biomass using vacuum filtration and sterilized by 0.45 µm cellulose acetate membrane syringe filters (Sarstedt Aktiengesellschaft & Co., Nümbrecht, Germany). A 5 mm agar plug of *B. cinerea* or *F. graminearum* was inoculated into 50 ml Erlenmeyer flask containing 10 ml of culture filtrate and incubated at 25 °C with constant shaking (100 rpm). Plant pathogenic fungi inoculated into

respective fresh culture media were used as control treatment. Mycelial biomass was harvested on 4 DAI using vacuum filtration and dried overnight at 65 °C. Mycelial biomass production was determined by measuring mycelial dry weight.

3.9 Barley bioassay for *F. graminearum*

An *in vivo* bioassay for *F. graminearum* foot rot disease on barley was performed using a sand seedling test as described previously (Jensen *et al.*, 2007; Dubey *et al.*, 2014a). In brief, barley seeds were surface-sterilized with 2% NaOCl and air-dried on the benchtop of a laminar air-flow. The seeds were then incubated with the conidial suspensions (5×10^7 conidia/ml in water) of *C. rosea* WT, or deletion strains for 30 minutes in a rotary shaker (150 rpm). Seeds incubated with sterile distilled water were used as control. The seeds were sown in pre-wetted sand in the 5×5×5 cm plastic pots (3 seeds/pot) (Figure 2). For pathogen inoculation, a 5 mm agar plug of 15 days old *F. graminearum* mycelium were placed close to the seeds on the plastic pot. PDA plug without *F. graminearum* was used as control. Thereafter, the seeds along with PDA plugs were covered with moist sand. The experiment was performed in five biological replicates with 15 plants per replicate. The seeds were then grown in a growth chamber with a photoperiod of 12 h light (150 µmol m^2 s^{-1} light intensity)/12 h dark, 70% ± 5% relative humidity, and 15 ± 1°C temperature (Dubey *et al.*, 2014a). Seedlings were harvested 3 weeks post-inoculation, and disease symptoms were scored on a 0 to 4 scale: 0 = healthy plants with no symptoms, 1 = slightly brown roots and coleoptiles, 2 = moderately brown roots and coleoptiles, 3 = severely brown roots and coleoptiles, and 4 = dead plants (Jensen *et al.*, 2007; Dubey *et al.*, 2014a).

Figure 2. In vivo barley bioassay of *C. rosea* against *F. graminearum* using a sand seedling test. The picture is the representative of the actual experimental set-up except the number of barley seeds in each pot. Three barley seeds, coated with *C. rosea* conidia were placed in the moist sand of each pot with a 5 mm *F. graminearum* agar plug. Photo: Mukesh Dubey

3.10 Statistical analysis

All the statistical analyses in this study were performed using Minitab 16 statistical software (Minitab, Inc.). Significant differences on gene expression and phenotype data were performed using one-way analysis of variance (ANOVA) and pairwise comparisons were made using Fisher's exact test with 95% level of confidence.

4 Results

4.1 Sequence analysis

The presence of different protein domains and their architectures in all 32 PKS in *C. rosea* was different from each other (Figure 3). Most of the non-reducing PKSs (PKS1, PKS2, and PKS4) have the similar domain architecture. On the other hand, reducing PKSs (PKS5-PKS31) were found to have very diverse domain architectures even in the same group members (Figure 3).

4.2 Expression analysis of *pks*

Gene expression analysis showed a significant induction in expression of 19 *pks* (*pks1. Pks2, pks6-pks10, pka12-pks17, pks20-pks22, pks24, pks26,* and *pks28-pks30*), a significant suppression of *pks22* during pigmentation in comparison to control (Figure 4 A, Table S1). Expression of 11 *pks* (*pks3-pks5, pks11, pks18, pks19, pks23, pks25, pks27, pks31,* and *pks32*) was not detected under this condition (Figure 4 A, Table S1). During confrontation of *C. rosea* with plant pathogenic fungus *B. cinerea* (Cr-Bc), expression of 18 *pks* (*pks1-pks4, pks8, pks10, pks12-pks16, pks17, pks19, pks20, pks22,* and *pks28-pks30*) was significantly induced as compared with control (Cr-Cr) or *C. rosea* and *F. graminearum* confrontation (Cr-Fg), while expression of 6 *pks* (*pks5, pks11, pks18, pks25, pks27,* and *pks32*) was not detected under this condition (Figure 4 B, Table S1). When *C. rosea* confronted with *F. graminearum*, 7 *pks* (*pks2, pks4, pks8, pks16, pks22, pks29,* and *pks31*) were significantly up-regulated and 15 *pks* (*pks1-pks4, pks9, pks10, pks12, pks13, pks15-pks17, pks19, pks20, pks28* and *pks30*) were significantly downregulated as compared with Cr-Cr or Cr-Bc (Figure 4 B, Table S1).

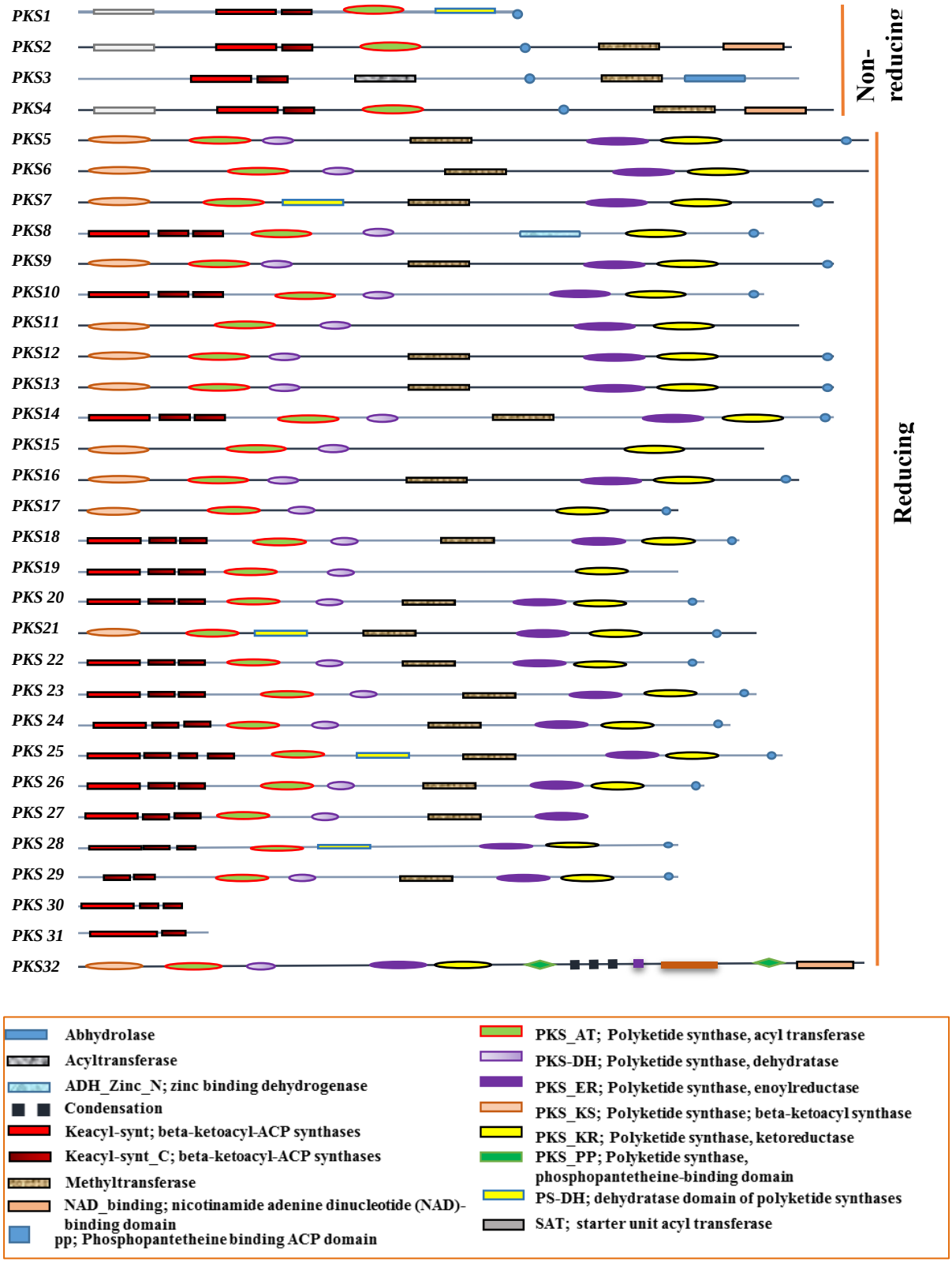

PKS1
PKS2
PKS3
PKS4
PKS5
PKS6
PKS7
PKS8
PKS9
PKS10
PKS11
PKS12
PKS13
PKS14
PKS15
PKS16
PKS17
PKS18
PKS19
PKS 20
PKS21
PKS 22
PKS 23
PKS 24
PKS 25
PKS 26
PKS 27
PKS 28
PKS 29
PKS 30
PKS 31
PKS32
Non-reducing
Reducing
Abhydrolase
Acyltransferase
ADH_Zinc_N; zinc binding dehydrogenase
Condensation
Keacyl-synt; beta-ketoacyl-ACP synthases
Keacyl-synt_C; beta-ketoacyl-ACP synthases
Methyltransferase
NAD_binding; nicotinamide adenine dinucleotide (NAD)-binding domain
pp; Phosphopantetheine binding ACP domain
PKS_AT; Polyketide synthase, acyl transferase
PKS-DH; Polyketide synthase, dehydratase
PKS_ER; Polyketide synthase, enoylreductase
PKS_KS; Polyketide synthase; beta-ketoacyl synthase
PKS_KR; Polyketide synthase, ketoreductase
PKS_PP; Polyketide synthase, phosphopantetheine-binding domain
PS-DH; dehydratase domain of polyketide synthases
SAT; starter unit acyl transferase

Figure 3. Schematic representation of domain architectures of 32 PKS of *C. rosea* IK726. Illustration was based on the alignment of protein domains using SMART protein analysis tool. The architectures of different domains were reflected by different coloured shape and is not co-related with the actual length, size and shape.

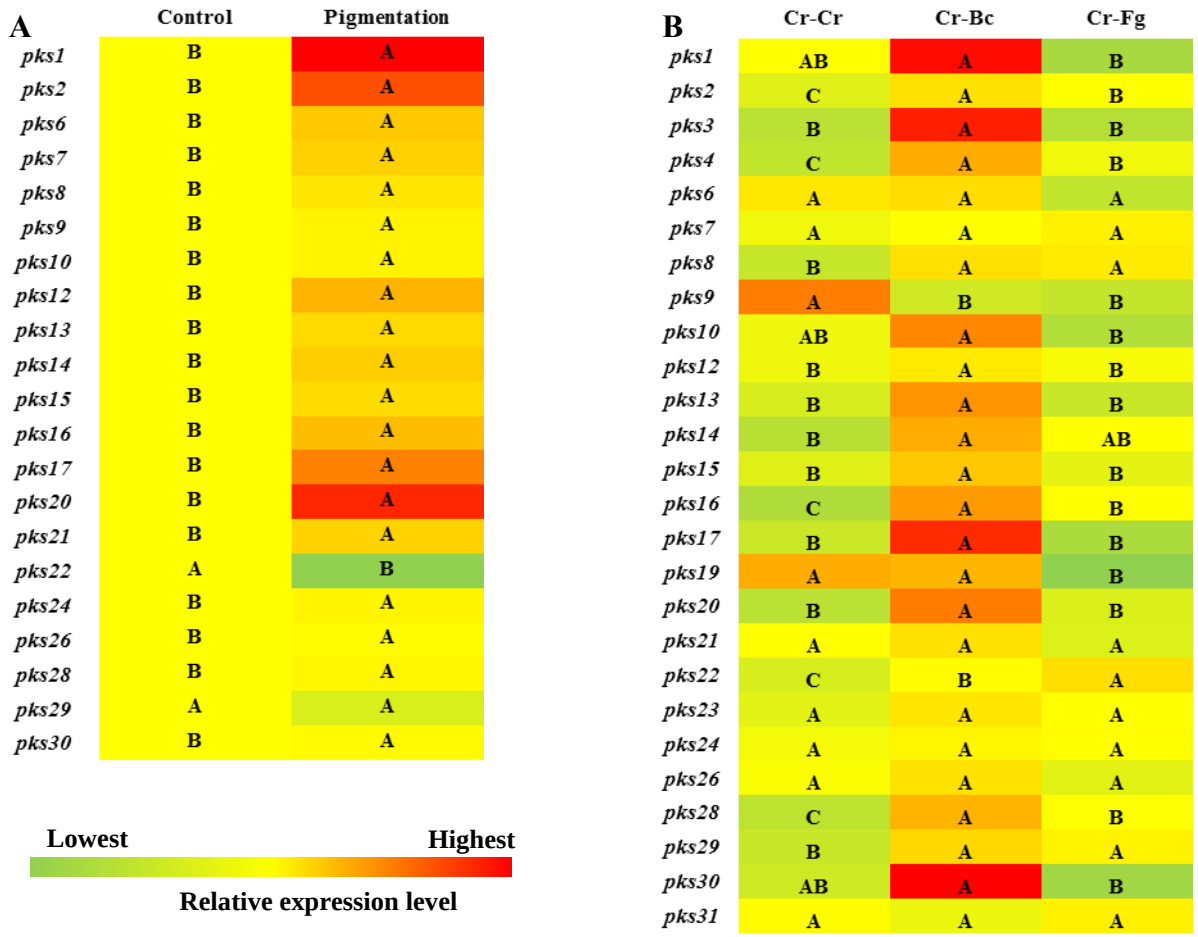
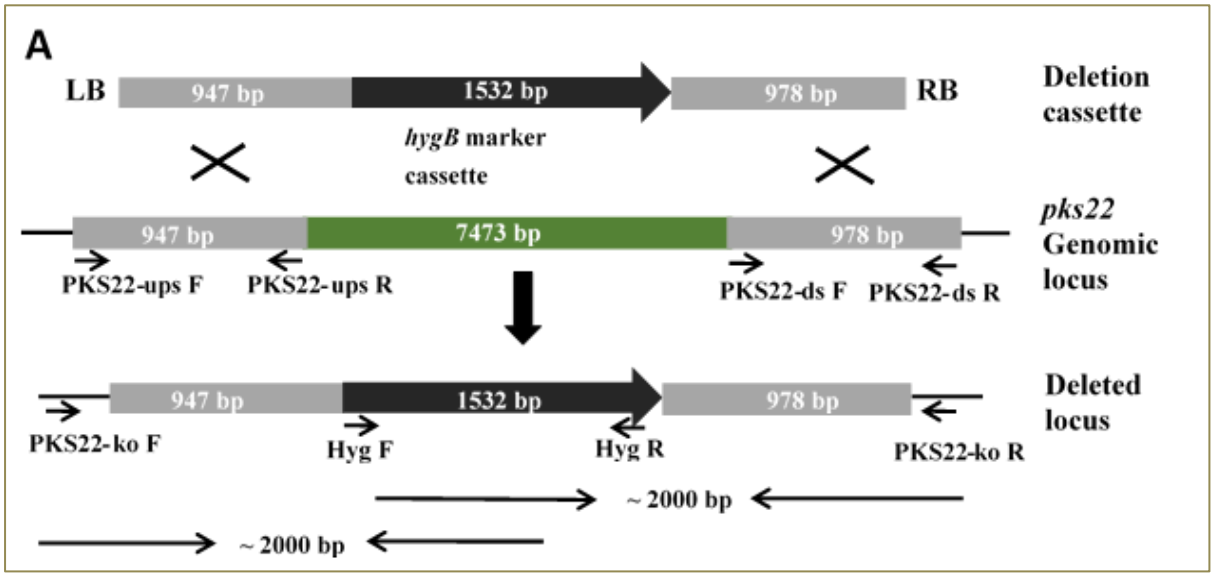


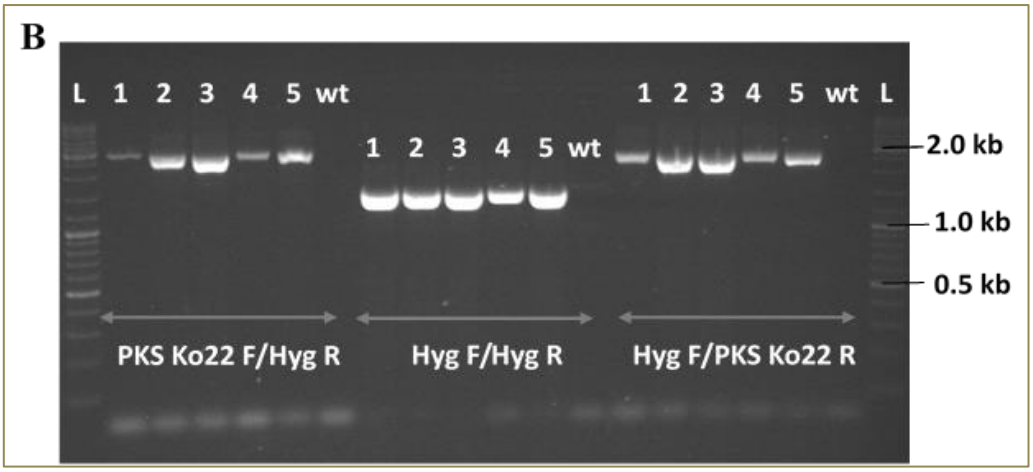
Figure 4. Relative expression of *pks* in *C. rosea* during pigmentation and during interaction with pathogenic fungi. Relative expression of 32 *pks* was performed using RT-qPCR during pigmentation (A) and during antagonistic interaction of *C. rosea* with itself (Cr-Cr), *B. cinerea* (Cr-Bc) or *F. graminearum* (Cr-Fg) (B). The relative expression value of *pks* was standardized using log transformation, mean centering, and auto-scaling. Different colour codes indicate the standardized relative expression levels. Statistically significant difference ($P \leq 0.05$) in gene expression between treatments is indicated by different letters.

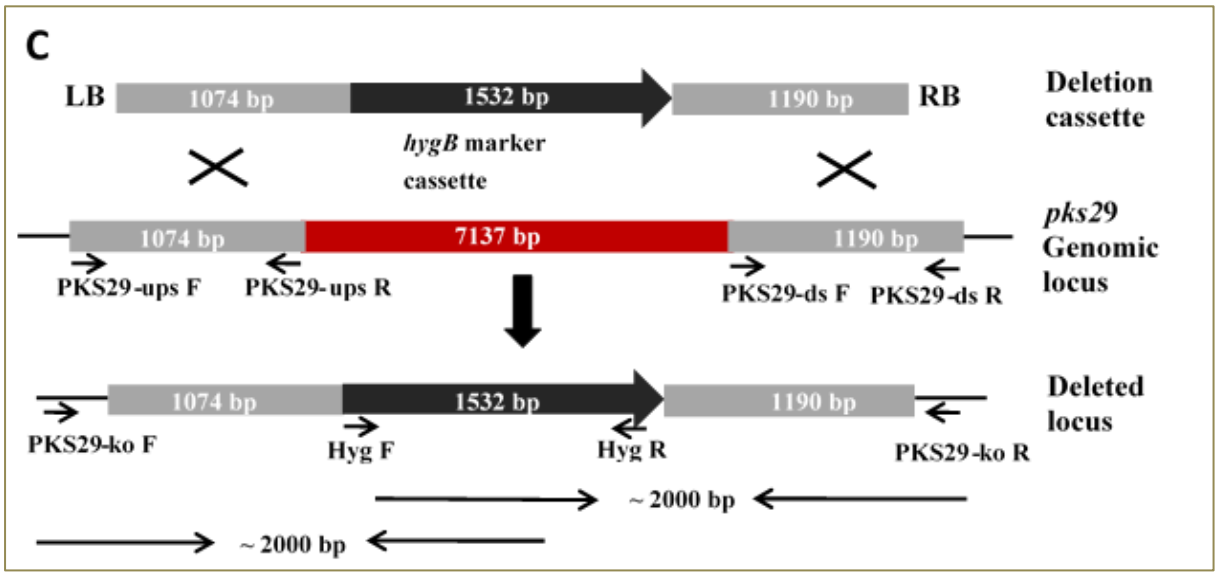
4.3 Generation of gene deletion mutants

Transformation of *C. rosea* generated 50-200 putative transformants for each gene after two rounds of selection on plates containing 200 µl/ml of hygromycin B (Figure 5). In order to identify positive gene deletion mutants, PCR reaction using the primers pairs schematically presented in Figure 6 was done. Expected PCR products were obtained from positive transformants whereas no PCR product was amplified in WT, which indicates the successful deletion of the target gene in the *C. rosea* genome (Figure 6). Five PCR positive transformats out of 72 screened for *pks22* deletion, and 2 PCR positive transformants out of 32 screened for *pks29* deletion were found. RT-PCR on cDNA from positive deletion mutants along with the WT using the *pks22* and *pks29* specific primers demonstrated the lack of transcripts in any of the mutant strains (Figure not shown). PCR screening of hygromycin positive transformants for *pks1*, *pks2*, *pks3*, and *pks4* (more than 100 colonies were screened for individual gene) did not produce desired product suggesting ectopic insertion of deletion cassette.

Figure 5. Agrobacterium tumefaciens-mediated transformants







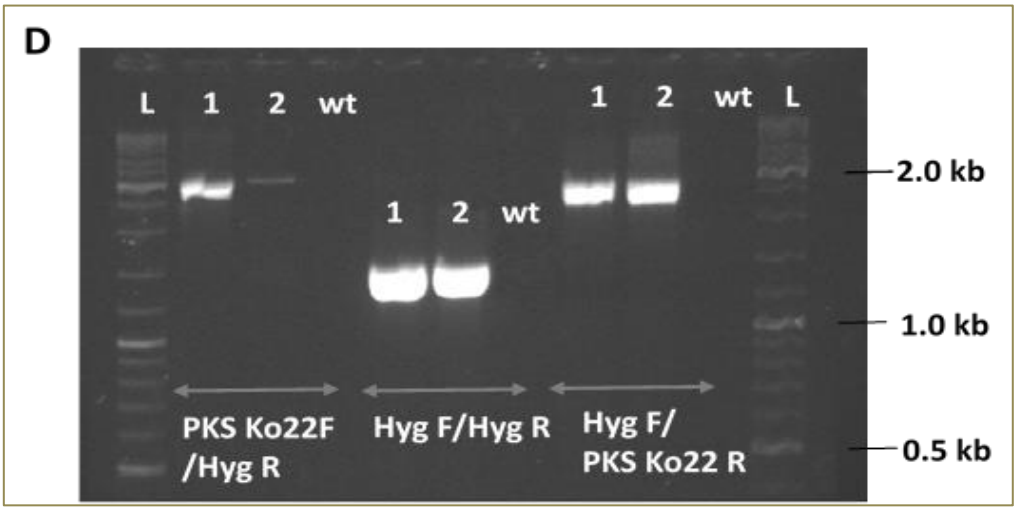


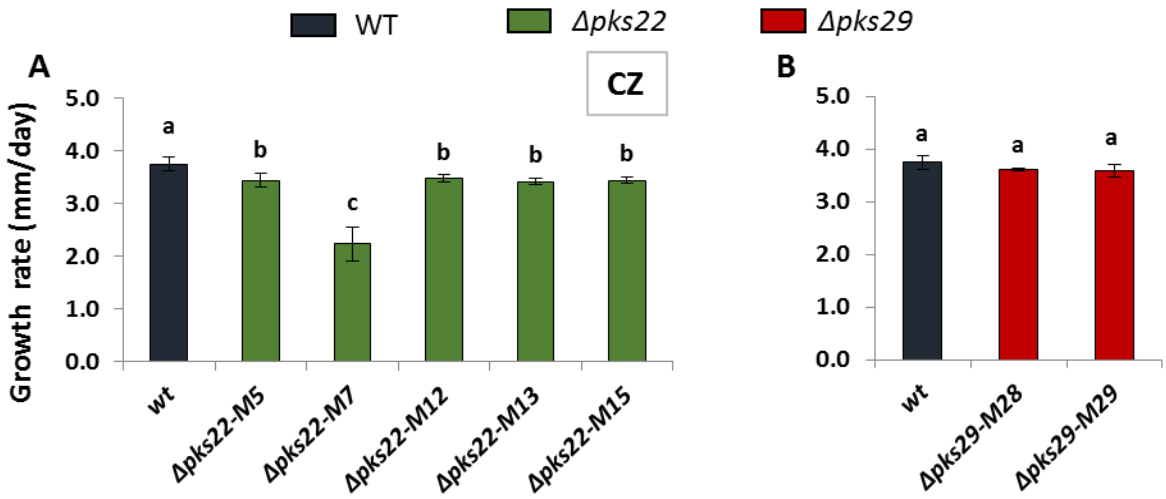
Figure 6. Schematic representation of the deletion cassettes and validation of deletion mutants.

A & C. Positions of *pks22* (A) and *pks29* (C) in genomic locus and in deletion cassettes. The coding regions of *pks22* and *pks29* were replaced by *hygB* cassette by homologous recombination to generate *Δpks22* and *Δpks29*.

The location of primers, used to construct the deletion cassette and to validate mutants using PCR is indicated by arrow heads. LB=left border; RB=Right border. **B & D.** Validation of *Δpks22* and *Δpks29* using primer pairs PKS22/PKS29 Ko F/Hyg R, Hyg F/Hyg R and Hyg F/PKS22/PKS29 Ko R. PKS22/PKS29 Ko F/Hyg R amplified the whole *hygB* cassette and the upstream region along with some outside part of the deletion cassette generating ~2 kb PCR product. Hyg F/Hyg R generated ~1.5 kb amplified product while amplifying the whole *hygB* cassette. Hyg F/PKS22/PKS29 Ko R amplified the whole *hygB* cassette and the downstream region along with some outside part of the deletion cassette generating~2.0 kb PCR product. L, DNA ladder; WT, wild type; 1-5, independent deletion strains.

4.4 Deletion of *pks22* in *C.rosea* IK726 results in reduced mycelial growth

Five independent *pks22* deletion mutants (*Δpks22-M5, Δpks22-M7, Δpks22-M12, Δpks22-M13,* and *Δpks22-M15*) and two independent deletion mutants of *pks29* (*Δpks29-M28* and *Δpks29-M29*) along with WT *C. rosea* strain were used in all phenotypic analysis. A significant reduction in mycelial growth of *Δpks22* was recorded compared with WT both in PDA ($P \leq 0.004$) and CZ-agar ($P \leq 0.000$) medium (Figure 7. A & C). However, no significant difference in mycelial growth rate was observed in between WT and *Δpks29* either in PDA or CZ medium (Figure 7 B & D).



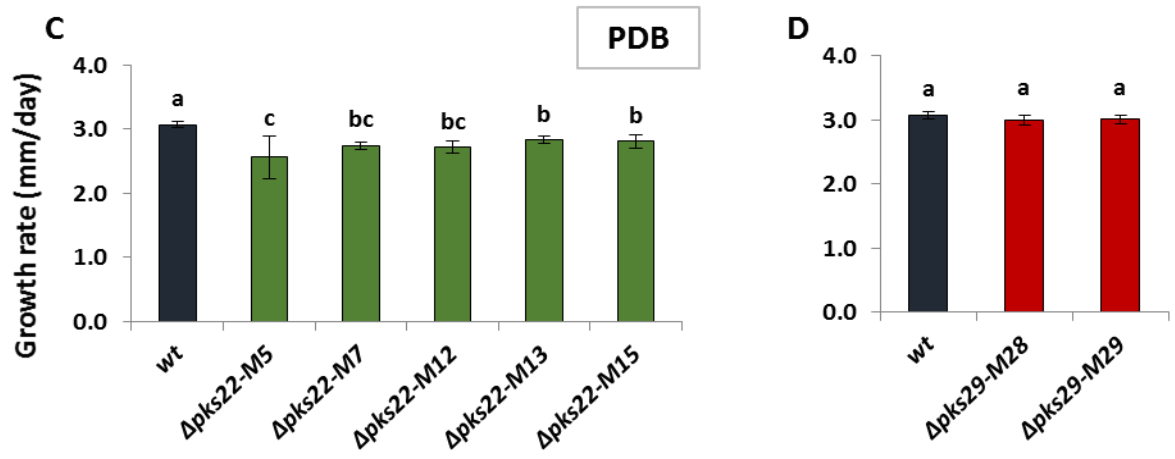
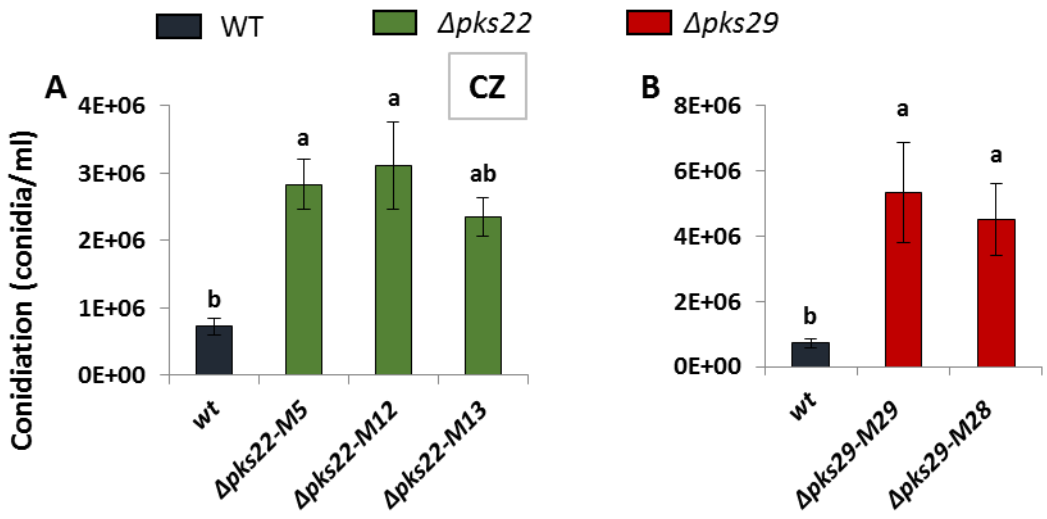


Figure 7. **Growth rate of *C. rosea* WT and deletion mutants on Czapek-Dox (CZ) agar and Potato Dextrose Agar (PDA) medium**. Mycelia of WT (A, B, C, D), *Δpks22* (A, C) or *Δpks29* (B, D) mutants were grown on CZ agar (A, B) or PDA (C, D) plate and incubated at 25°C. Growth rate was recorded on 5 days post inoculation. Error bars represent standard deviation based on four biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiment based on Fisher's exact test.

4.5 Deletion of *pks* results in high conidiation rate and the effect is culture media dependent

A significant increase in the conidiation was observed for both *Δpks22* ($P \leq 0.001$) and *Δpks29* ($P \leq 0.026$) strains in CZ medium as compared with WT (Figure 8. A & B), while no difference in the conidiation was detected in any of the mutants in PDB media as compared with WT (Figure 8. C & D).



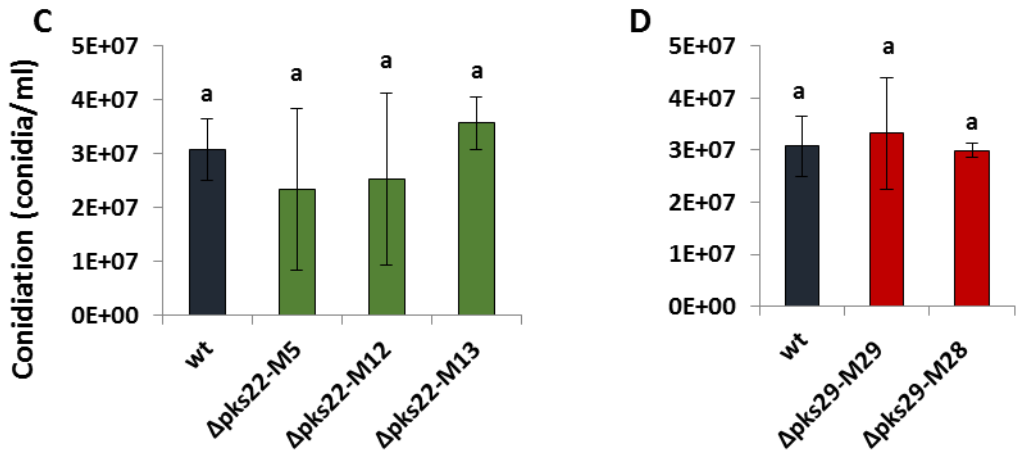
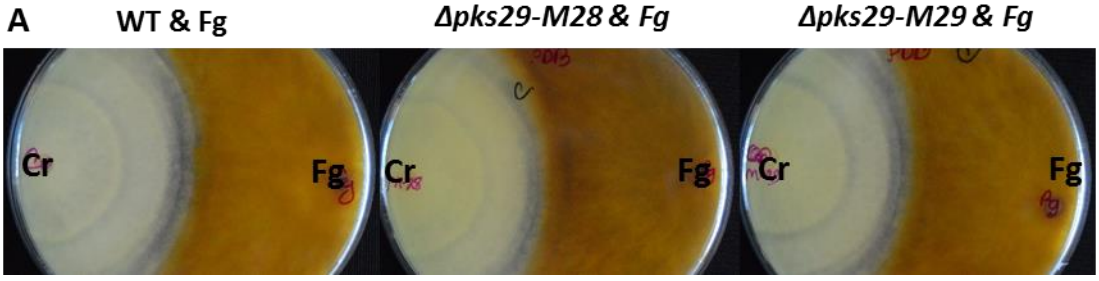


Figure 8. **Conidiation of WT and deletion mutants on Czapek-Dox (CZ) agar and Potato Dextrose Agar (PDA) medium.** Mycelia of WT (A, B, C, D), *Δpks22* (A, C) and *Δpks29* (B, D) mutants were grown on CZ agar (A, B) or PDA (C, D) plate and incubated at 25°C for 12 days in dark culture room, with the exposure of light in every twice day to induce conidiation. Conidial number was recorded using a bright line haemocytometer. Error bars represent standard deviation based on four biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on Fisher's exact test.

4.6 Deletion of *pks29* reduces antagonism capability of *C.rosea* against pathogenic fungi and the effect is culture media dependent

A significant decrease *(*$P \leq 0.011$*)* in length of the lytic zone was found in dual culture interaction between *Δpks29* and *F. graminearum* on PDA medium in comparison to interaction between WT and *F. graminearum* (Figure 9 A & C), while no difference in lytic zone was observed in dual culture interaction on PDA medium between *Δpks22* and *F. graminearum* compared with WT and *F. graminearum* strain (Figure 9 B). Similar dual culture interaction experiment against *A. alternata* or *B. cinerea* showed no significant difference in lytic zone on PDA medium (data not shown). In addition, dual culture interaction assay showed no significant difference in the ability to suppress the growth of *A. altarnata*, *B. cinerea* or *F. graminearum* between WT and deletion strains in CZ medium (data not shown).



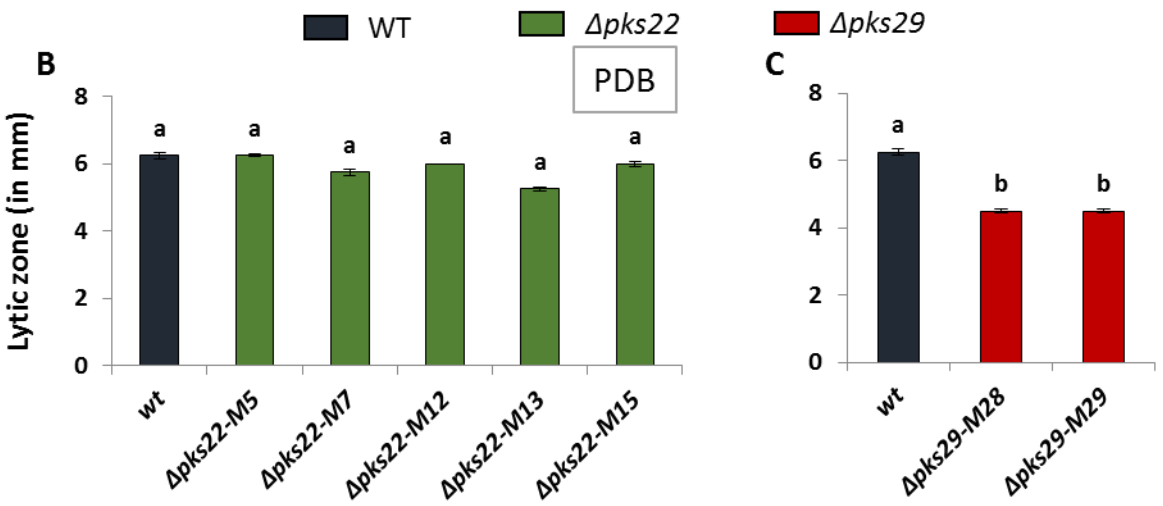
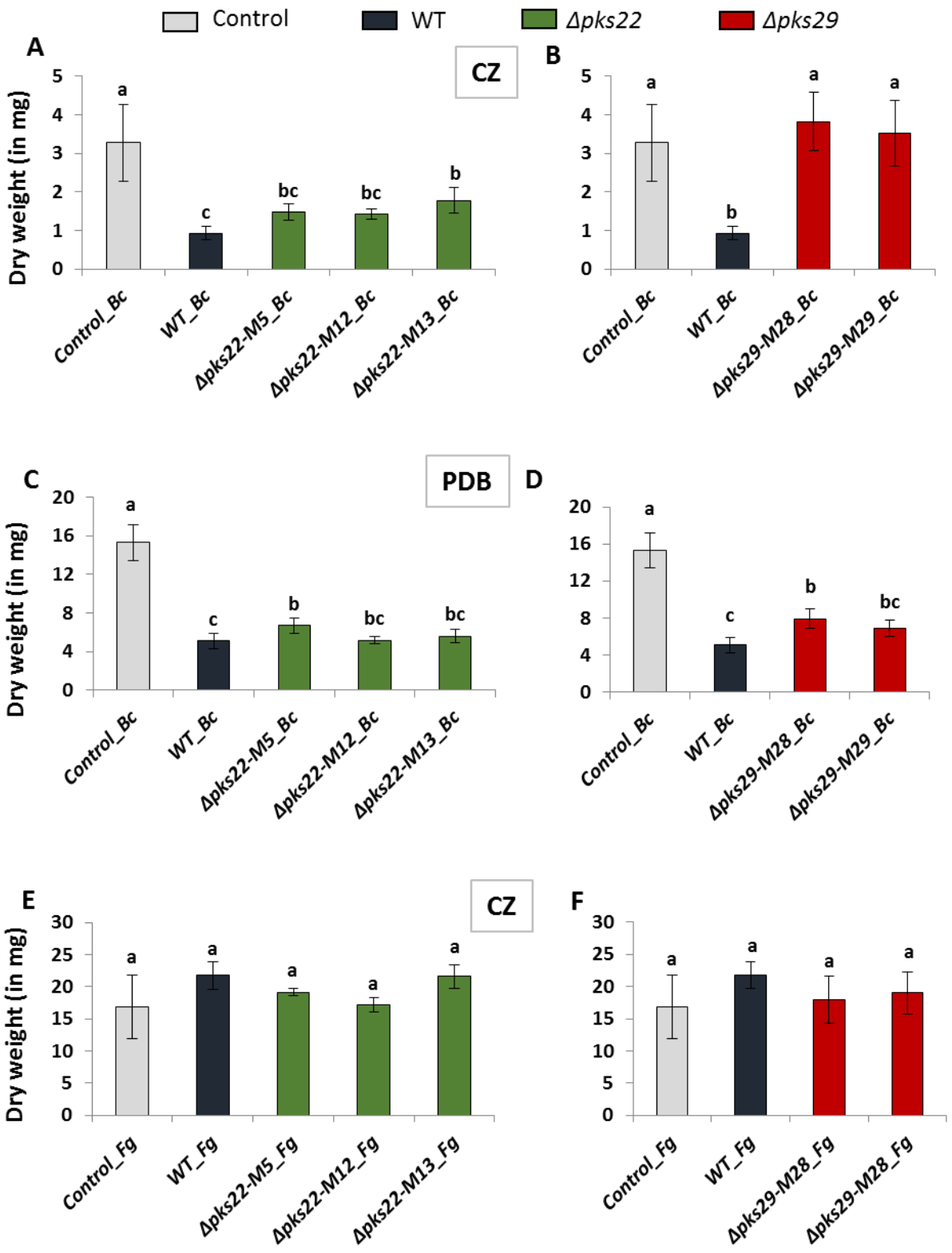


Figure 9. **In vitro plate confrontation assays of *C. rosea* WT and deletion mutants with *F. graminearum* in Potato Dextrose Agar (PDA) medium**. Agar plugs of *C. rosea* (Cr) strains and *F. graminearum* (Fg) were inoculated on opposite sides in a 9 cm PDA plate and incubated at 25°C in darkness. On 15 days post inoculation, the length of the lytic zone was measured in millimetre (mm). The experiment was carried out in four biological replicates. Error bars represent standard deviation based on biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on Fisher's exact test.

Secretion assay in CZ medium showed significant difference ($P \leq 0.001$) in biomass production of *B. cinerea* grown in Δ*pks29* strain culture filtrate (Figure 10 B) but did not show significant difference in Δ*pks22* strain culture filtrate (except Δ*pks22-M13*) compared to WT (Figure 10 A). A similar experiment in PDB medium showed similar result (except *Δpks29-M29*) (Figure 10 C & D). In contrary to secretion assay against *B. cinerea*, similar experiment against *F. graminearum* did not show significant difference in *F. graminearum* biomass production in culture filtrates of Δ*pks29* or Δ*pks22* strains in comparison to WT (Figure 10 E, F, G & H). Interestingly, no significant reduction in *F. graminearum* biomass in CZ culture filtrates was found in comparison to control CZ medium (Figure 10 E & F)


Control
WT
Δpks22
Δpks29
A
CZ
B
Dry weight (in mg)
Control_Bc
WT_Bc
Δpks22-M5_Bc
Δpks22-M12_Bc
Δpks22-M13_Bc
Δpks29-M28_Bc
Δpks29-M29_Bc
C
PDB
D
E
CZ
F
Control_Fg
WT_Fg
Δpks22-M5_Fg
Δpks22-M12_Fg
Δpks22-M13_Fg
Δpks29-M28_Fg
Δpks29-M28_Fg

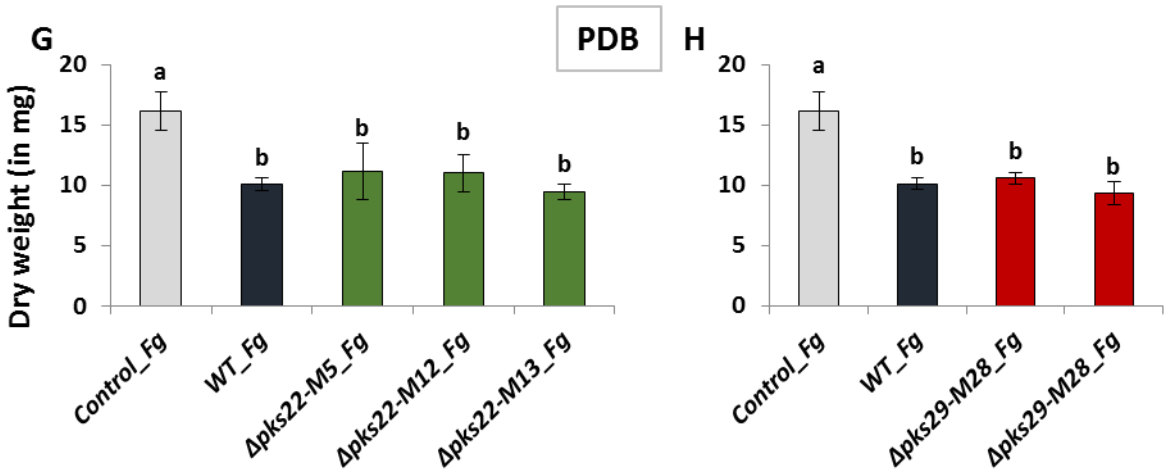


Figure 10. Secretion assay of *C. rosea* WT and deletion mutants. Fungal conidia of *C. rosea* WT, Δ*pks22* and Δ*pks29* were inoculated in Czapek-Dox broth (A, B, E, and F) or potato dextrose broth (C, D, G, and H) medium and incubated for 6 days at 25°C in a rotary incubator. Culture filtrates were collected after removing the mycelial mass and agar plugs of *B. cinerea* (Bc) or *F. graminearum* (Fg) were inoculated and grown at 25°C. Fungal biomass was harvested 4 days post inoculation, dried and measured. The experiments were carried out in four biological replicates. Error bars represent standard deviation based on biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on Fisher's exact test.

4.7 Deletion of *pks* does not have any effect to control *F. graminearum* foot rot disease in Barley

No difference in the biocontrol ability of *C. rosea* WT and deletion mutants to control *F. graminerum* foot rot disease was observed on barley plants in the sand seedling test (Figure 11).

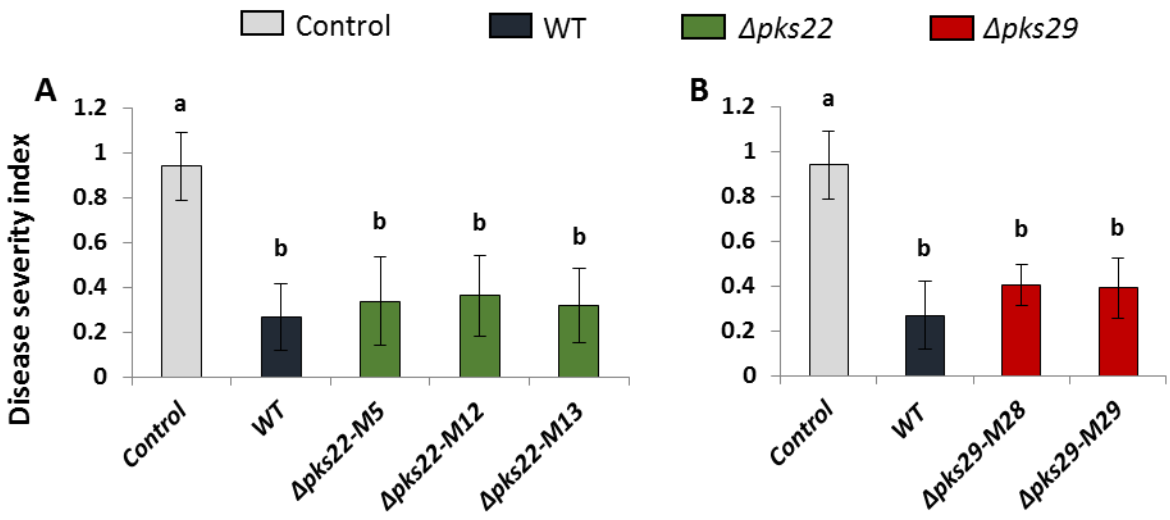


Figure 11. In vivo bioassay of *C. rosea* WT and deletion mutants against *F. graminearum* foot rot disease on barley. Barley seeds were coated with *C. rosea* conidial suspension (5×10^7conidia/ml), sown in the wet sand, and inoculated with *F. graminearum* agar plug. The seeds were allowed to grow until typical foot rot disease symptoms appeared. After 3 weeks of inoculation, seedlings were harvested and disease symptoms were scored on a 0 to 4 scale. The experiments were carried out in five biological replicates with 9 to 14 plants in each replicate. Error bars represent standard deviation based on biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on Fisher's exact test.

5 Discussion

5.1 Domain architecture

The domain analysis of 32 PKS in *C. rosea* reveals that, domain architecture differs according to the type of PKS. All the domains to synthesize aromatic PKS as for example SAT, KS, transferase are present in non-reducing PKS (Figure 3). According to the phylogenetic network (Figure 1), non-reducing PKS2, PKS3, and PKS4 belong to methylated monocyclic aromatic group which is in the line of our domain analysis. Fungal reducing polyketides are very diverse because of their number and modes of reduction reaction (Keller *et al.*, 2005). In our study, among the different groups of reducing polyketides (Figure 1), all the PKSs of fumonisin group show similar domain architecture (Figure 3) but reducing PKSs in lovastatin diketides (Figure 1) are diverse in association with their domain architecture (Figure 3). The diversity in domain architecture among reducing *C. rosea* PKSs is consistent with this aforementioned statement.

5.2 Expression analysis

In fungi, PKSs are generally responsible for their ecological and evolutionary adaptation (Fujii, 2010). The involvement of PKS in fungal mycoparasitism, pigmentation and stress resistance is shown in biocontrol fungus *T. reesei* (Atanasova *et al.*, 2013). *C. rosea* genome possess significantly higher number of genes, coding for PKS than biocontrol fungus *Trichoderma* (Karlsson *et al.*, 2015). The expansion of PKS encoding genes indicates the role of polyketides in antagonism, biocontrol activity and pigmentation in *C. rosea*. This is supported by significant induced expression of several *pks* genes during pigmentation and antagonistic interaction with plant pathogenic fungi.

In the previous studies, it was examined that non-reducing PKSs are responsible for mycelial and conidial pigmentation (Gaffoor *et al.*, 2005; Pihet *et al.*, 2009; Atanasova *et al.*, 2013). The observation regarding relative expression of *pks* genes (*pks1, pks2, pks3* and *pks4*) encoding non-reducing PKSs during pigmentation is in the line of these previous studies. In our gene expression study, *pks1* and *pks2* had the highest induction during pigmentation, though most of the *pks*s were highly induced during pigmentation. Interestingly, no detection in *pks3* and *pks4* expression from control mycelium but significant amount of fluorescence detection from pigmented mycelial cDNA indicates the importance of PKS in pigmentation in *C. rosea*. During antagonistic interaction of *C. rosea* with *B. cinerea*, both reducing and non-reducing *pks*s have been induced which is also consistent with the previous studies, where the expression

of both reducing and non-reducing PKSs was detected during mycoparasitism in biocontrol fungus *T. reesei* and *T. harzianum* (Atanasova *et al.*, 2013; Yao *et al.*, 2016). Furthermore, gene expression analysis during fungal-fungal interactions indicates the pathogen specific *pks* expression in *C. rosea*.

5.3 Functional characterization of PKS

It was not possible to characterize all 32 PKS within the limited time period in a master's project. Therefore, we selected 6 PKS encoding genes based on their regulatory gene expression pattern in different culture medium (Umma Fatema, research training project; 15 hp), during pigmentation, during fungal-fungal interactions as well as based on their phylogenetic network with functionally characterized PKS of other ascomycetes. However, only four PKSs (PKS1-PKS4) belong to the non-reducing type. Therefore it was possible to select such a group with small members to characterize the function for the whole group and we hypothesized that these non-reducing PKSs are mainly involved in *C. rosea* pigmentation. Among the reducing *pks*s, only 2 *pks*s (*pks22* and *pks29*) have been selected because both of the *pks*s upregulated during antagonistic interaction with pathogenic fungi but down-regulated during pigmentation in comparison to control. We hypothesized that they could be involved in antagonism but not in pigmentation. To determine the function of PKS, each of the selected *pks* was deleted individually from the WT *C. rosea* genome and the morphology, and mycoparasitic/antagonistic ability of each of the mutants were examined in comparison to the WT.

In this study, loss of function of *pks22* led to a reduced mycelial growth in *C. rosea.* The involvement of *pks* in mycelial growth of several fungi has previously been shown. For instance, in *F. graminearum* , disruption of *GRS1* and *PKS2* inhibited mycelial growth (Gaffoor *et al.*, 2005). Similarly, *flup* disruption mutants in *A. parasiticus* had significant reduction in mycelial growth and sporulation, and significant reduction of aflatoxin production (Zhou *et al.*, 2000). However, no difference in mycelial growth rate in *pks29* deletion mutants in comparison to WT suggests differential role of PKS29. Our results did not show any role of *pks22* and *pks29* in pigmentation. Both WT and deletion mutants of respective genes were similar in pigment production. No difference in pigmentation was consistent with the relative gene expression of *pks22* and *pks29* where expression of both the genes was down-regulated during pigmentation as compared with control.

Disruption of *pks22* and *pks29* results in high conidiation in CZ-agar medium which may suggest their involvement in conidiation in *C. rosea*. In *S. macrospora*, both loss and over-expression of *pks4* resulted in disrupted sexual development (Schindler & Nowrousian, 2014). In fungus *T. reesei*, *pks4* also showed its impact on conidiation and conidial cell wall stability. In the study of Atanasova *et al.*, (2013), loss of *pks4* resulted in loss of green conidial pigmentation and increased sensitivity to UV light. These previous studies and our findings can indicate that constant expression of certain *pks*s is required for normal sexual development of fungi. The plausible explanation for more conidiation in *pks22* or *pks29* deletion mutants in *C. rosea* is that the deletion mutants either spend its physiological costs for conidiation which otherwise are responsible for production of secondary metabolites or disruption of a polyketide biosynthesis pathway resulted in accumulation of intermediates and/or other metabolites which in turn prompted conidiation. The increased conidiation on CZ medium but not on PDA medium supports the fact that there is a relationship between conidiation and disturbance in metabolite production/accumulation.

A dual culture test and secretion assay were performed to compare the antagonistic ability between WT and *pks* deletion strains. Reduction in antagonism against *F. graminearum* in *pks29* deletion strains in interaction during dual culture assay suggests a role of PKS29 in antagonism. However, no difference in antagonism against in secretion assay suggest the production of toxic metabolites toxic only during living interaction with *F. graminearum*. In contrast, an opposite result was obtained against *B. cinerea* on CZ medium when loss of *pks29* failed to inhibit the growth of *B. cinerea* in CZ-culture filtrate but not during dual culture interaction on CZ agar plate. These data suggest a role of PKS29 in biosynthesis of metabolite/s toxic to *F. graminearum* and *B. cinerea*. In contrast to dual culture test result, no significant difference in fusarium foot rot on barley between WT and *pks29* deletion strains indicates the functional complementation of PKS29 during three way interactions among *C. rosea*, *F. graminearum,* and barley. However, this experiment needs to be repeated as there was no proper disease development even in control treatment. The role of *pks* in antagonism is shown in previous studies specifying the involvement of *pks4* in reduced antagonistic potential against pathogenic fungi (Atanasova *et al.*, 2013)

5.4 Functional diversity of PKS in *C. rosea* under diverse culture conditions

The nutritional conditions such as source of carbon (C), nitrogen (N_2), and pH of the culture media can affect the synthesis of secondary metabolites in fungi and bacteria (Bode *et al.*,

2002). In my research training project, there were significant differences in gene expression of 28 *pks* in *C. rosea* grown in 5 different culture media (different in terms of their C and N_2 source, and pH). The differences in gene expression were positively related with the antagonism of *C. rosea* against plant pathogenic fungi *A. alternata, B. cinerea, F. graminearum,* and *R. solani*. Culture medium dependent phenotypic effects in *pks22* and *pks29* deletion strains in the present study may be related with the differences in carbon and nitrogen source and pH between CZ (3% sucrose, 0.2% NaNO3, pH 7.3 ±0.2) and PDA (2% dextrose, 0.4% potato starch, pH 7.3 ± 0.2) medium.

6 Conclusion

From the expression analysis of 32 *pks* in *C. rosea,* we can conclude that genes encoding non-reducing PKSs have higher relatedness over reducing PKSs in terms of *C. rosea* pigmentation. Our phenotypic analysis of deletion mutants suggests that PKSs in *C. rosea* are involved in antagonism against plant pathogenic fungi *B. cinerea* and *F. graminearum*. This study also proposes biological functions of PKS in mycelial growth and conidiation of *C. rosea*. Our results also suggest that production of PKS-derived compounds toxic for *B. cinerea* or *F. graminearum* depend on nutritional status and pH of the growing media.

7 Future prospect

a. Our works offer a good framework for comparative study on conidial germination under different abiotic stresses conditions in *C. rosea* WT and mutants' background to study a role of PKS in abiotic stress tolerance in *C. rosea*.
b. More work can be done in the direction of biochemical investigation of secreted products in *C. rosea* culture filtrate both in WT and deletion mutants to identify the polyketides specific for pigmentation and antagonism in *C. rosea.*
c. One can also analyse the expression of other *pks*s in deletion mutants to see whether gene deletion affects the expression pattern of other *pks* during antagonistic interaction or pigmentation in *C. rosea.*

8 Acknowledgement

All praise to almighty Allah. The last eight months of my master's thesis was a period of intense learning period for me to make a new outlook not only in the scientific arena but also on a personal level.

I would like to convey my endless gratitude to Mukesh to be my supervisor. Thank you for your patient guidance when it was time to make me perfect for this project from very zero. Without your kind help, it was not possible to walk in this long journey very smoothly.

I would like to express my appreciation to my co-supervisors Dan and Magnus for their valuable and constructive advice to furnish the planning and development of my master's project. Thank you, Dan, for giving me the opportunity to pursue my master's thesis in your group and for your support whatever I needed. Thank you, Magnus, for your help during data analysis and useful critiques to structure this project. In our every discussion, I just wondered how a person can think of such different logical aspects.

I consider it an honour to have Malin as my examiner. Whenever I talked to you, I ended up with a feeling that I have a shelter to relief my stress.

Eddy, it will be really short if I want to express your endless support in the lab in the word. In any of my problems, you always had a solution or suggestion for me within a second.

I also like to extend my appreciation to Katarina, Rena, and Maria for your technical supports in the lab. I am going to miss your smile to start an inspiring morning. Special thanks to Rajiv dada for your motivating suggestions.

I am also indebted to the friendships of Alvaro, Hernan and Laura. It was always enjoyable having dinner and fika together.

Thanks to all the Mycopat members. It was really a great time with you.

Abbu and Ammu, Allah does not give any words to acknowledge your sacrifices. You are always my dream-makers. Abbu, I am now on the way to fulfil your dream. Abba and Amma, your special care from 4000 miles away always motivates me to do something right. Sadhona apu, Borna, Tofayel, you do not know how much rhythm I get when you say 'Don't worry, everything will be ok', when there is a conflict between my personal and academic life.

My study in abroad remained a dream without my husband, Foteh. Because of you, I always felt relaxed inside during my thesis period. I know, there is always you to share all of my pains and stresses.

9 References

Ahmed, M., Laing, M. D. & Nsahlai, I. V. (2014). Use of Clonostachys rosea against sheep nematodes developing in pastures. *Biocontrol Science and Technology*, 24(4), pp 389–398.

Atanasova, L., Knox, B. P., Kubicek, C. P., Druzhinina, I. S. & Baker, S. E. (2013). The polyketide synthase gene pks4 of Trichoderma reesei provides pigmentation and stress resistance. *Eukaryotic Cell*, 12(11), pp 1499–1508.

Beltrán-García, M. J., Prado, F. M., Oliveira, M. S., Ortiz-Mendoza, D., Scalfo, A. C., Pessoa, A., Medeiros, M. H. G., White, J. F. & Di Mascio, P. (2014). Singlet Molecular Oxygen Generation by Light-Activated DHN-Melanin of the Fungal Pathogen Mycosphaerella fijiensis in Black Sigatoka Disease of Bananas. *PLoS ONE*, 9(3).

Bladt, T. T., Frisvad, J. C., Knudsen, P. B. & Larsen, T. O. (2013). Anticancer and antifungal compounds from Aspergillus, Penicillium and other filamentous fungi. *Molecules (Basel, Switzerland)*, 18(9), pp 11338–11376.

Bode, H. B., Bethe, B., Höfs, R. & Zeeck, A. (2002). Big effects from small changes: possible ways to explore nature's chemical diversity. *Chembiochem: A European Journal of Chemical Biology*, 3(7), pp 619–627.

Brown, D. W. & Salvo, J. J. (1994). Isolation and Characterization of Sexual Spore Pigments from Aspergillus nidulans. *Applied and Environmental Microbiology*, 60(3), pp 979–983.

Campbell, C. D. & Vederas, J. C. (2010). Biosynthesis of lovastatin and related metabolites formed by fungal iterative PKS enzymes. *Biopolymers*, 93(9), pp 755–763.

Chatterton, S., Jayaraman, J. & Punja, Z. K. (2008). Colonization of cucumber plants by the biocontrol fungus Clonostachys rosea f. catenulata. *Biological control : theory and application in pest management* [online],. Available from: http://agris.fao.org/agris-search/search.do?recordID=US201300915269. [Accessed 2017-04-09].

Chatterton, S. & Punja, Z. K. (2010). Factors influencing colonization of cucumber roots by Clonostachys rosea f. catenulata, a biological disease control agent. *Biocontrol Science and Technology*, 20(1), pp 37–55.

Dubey, M. K., Jensen, D. F. & Karlsson, M. (2014). An ATP-binding cassette pleiotropic drug transporter protein is required for xenobiotic tolerance and antagonism in the fungal biocontrol agent Clonostachys rosea. *Molecular plant-microbe interactions: MPMI*, 27(7), pp 725–732.

Dubey, M., Jensen, D. F. & Karlsson, M. (2016). The ABC transporter ABCG29 is involved in H2O2 tolerance and biocontrol traits in the fungus Clonostachys rosea. *Molecular genetics and genomics: MGG*, 291(2), pp 677–686.

Dvorska, J. E., Surai, P. F., Speake, B. K. & Sparks, N. H. (2001). Effect of the mycotoxin aurofusarin on the antioxidant composition and fatty acid profile of quail eggs. *British Poultry Science*, 42(5), pp 643–649.

Engh, I., Nowrousian, M. & Kück, U. (2007). Regulation of melanin biosynthesis via the dihydroxynaphthalene pathway is dependent on sexual development in the ascomycete Sordaria macrospora. *FEMS Microbiology Letters*, 275(1), pp 62–70.

Fakruddin, M., Chowdhury, A., Hossain, M. N. & Ahmed, M. M. (2015). Characterization of aflatoxin producing Aspergillus flavus from food and feed samples. *SpringerPlus* [online], 4. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4394062/. [Accessed 2017-04-20].

Fujii, I. (2010). Functional analysis of fungal polyketide biosynthesis genes. *The Journal of Antibiotics*, 63(5), pp 207–218.

Gaffoor, I., Brown, D. W., Plattner, R., Proctor, R. H., Qi, W. & Trail, F. (2005). Functional Analysis of the Polyketide Synthase Genes in the Filamentous Fungus Gibberella zeae (Anamorph Fusarium graminearum). *Eukaryotic Cell*, 4(11), pp 1926–1933.

Gelderblom, W. C., Jaskiewicz, K., Marasas, W. F., Thiel, P. G., Horak, R. M., Vleggaar, R. & Kriek, N. P. (1988). Fumonisins--novel mycotoxins with cancer-promoting activity produced by Fusarium moniliforme. *Applied and Environmental Microbiology*, 54(7), pp 1806–1811.

Graziani, S., Vasnier, C. & Daboussi, M.-J. (2004). Novel Polyketide Synthase from Nectria haematococca. *Applied and Environmental Microbiology*, 70(5), pp 2984–2988.

Hashimoto, M., Nonaka, T. & Fujii, I. (2014). Fungal type III polyketide synthases. *Natural Product Reports*, 31(10), pp 1306–1317.

Hobbelen, P. H. F., Paveley, N. D. & Bosch, F. van den (2014). The Emergence of Resistance to Fungicides. *PLOS ONE*, 9(3), p e91910.

Horrigan, L., Lawrence, R. S. & Walker, P. (2002). How sustainable agriculture can address the environmental and human health harms of industrial agriculture. *Environmental Health Perspectives*, 110(5), pp 445–456.

Howard, R. J., Ferrari, M. A., Roach, D. H. & Money, N. P. (1991). Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proceedings of the National Academy of Sciences*, 88(24), pp 11281–11284.

Hue, A. G., Voldeng, H. D., Savard, M. E., Fedak, G., Tian, X. & Hsiang, T. (2009). Biological control of fusarium head blight of wheat with Clonostachys rosea strain ACM941. *Canadian Journal of Plant Pathology*, 31(2), pp 169–179.

Jensen, B., Knudsen, I. M. B., Madsen, M. & Jensen, D. F. (2004). Biopriming of Infected Carrot Seed with an Antagonist, Clonostachys rosea, Selected for Control of Seedborne Alternaria spp. *Phytopathology*, 94(6), pp 551–560.

Jensen, D. F., Knudsen, I. M. B., Beck, M. L., Mamarabadi, M., Hockenhull, J. & Jensen, B. (2007). Development of a biocontrol agent for plant disease control with special emphasis on the near commercial fungal antagonist Clonostachys rosea strain 'IK726'. *Australasian Plant Pathology*, 36(2), pp 95–101.

Johansen, A. (2005). Non-target effects of the microbial control agents Pseudomonas fluorescens DR54 and Clonostachys rosea IK726 in soils cropped with barley followed by sugar beet. *Soil Biology and Biochemistry*, 37(12), pp 2225–2239.

Karimi, M., De Meyer, B. & Hilson, P. (2005). Modular cloning in plant cells. *Trends in Plant Science*, 10(3), pp 103–105.

Karlsson, M., Durling, M. B., Choi, J., Kosawang, C., Lackner, G., Tzelepis, G. D., Nygren, K., Dubey, M. K., Kamou, N., Levasseur, A., Zapparata, A., Wang, J., Amby, D. B., Jensen, B., Sarrocco, S., Panteris, E., Lagopodi, A. L., Pöggeler, S., Vannacci, G., Collinge, D. B., Hoffmeister, D., Henrissat, B., Lee, Y.-H. & Jensen, D. F. (2015). Insights on the evolution of mycoparasitism from the genome of Clonostachys rosea. *Genome Biology and Evolution*, 7(2), pp 465–480.

Keller, N. P., Turner, G. & Bennett, J. W. (2005). Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews. Microbiology*, 3(12), pp 937–947.

Knudsen, I. M. B., Hockenhull, J. & Jensen, D. F. (1995). Biocontrol of seedling diseases of barley and wheat caused by Fusarium culmorum and Bipolaris sorokiniana: effects of selected fungal antagonists on growth and yield components. *Plant Pathology*, 44(3), pp 467–477.

Knudsen, I. M. B., Hockenhull, J., Jensen, D. F., Gerhardson, B., Hökeberg, M., Tahvonen, R., Teperi, E., Sundheim, L. & Henriksen, B. (1997). Selection of biological control agents for controlling soil and seed-borne diseases in the field. *European Journal of Plant Pathology*, 103(9), pp 775–784.

Krauss, U., Ten Hoopen, M., Rees, A. R., Stirrup, T., Argyle, T., George, A., Arroyo, C., Corrales, E., Casanoves, F. & web-support@bath.ac.uk (2013). Mycoparasitism by Clonostachys byssicola and Clonostachys rosea on Trichoderma spp. from cocoa

(Theobroma cacao) and implication for the design of mixed biocontrol agents. *Biological Control*, 67(3), pp 317–327.

Lahlali, R. & Peng, G. (2014). Suppression of clubroot by Clonostachys rosea via antibiosis and induced host resistance. *Plant Pathology*, 63(2), pp 447–455.

M⊘ller, K., Jensen, B., Andersen, H. P., Stryhn, H. & Hockenhull, J. (2003). Biocontrol of Pythium tracheiphilum in Chinese Cabbage by Clonostachys rosea under Field Conditions. *Biocontrol Science and Technology*, 13(2), pp 171–182.

Mamarabadi, M. (2008). Real-time RT-PCR expression analysis of chitinase and endoglucanase genes in the three-way interaction between the biocontrol strain Clonostachys rosea IK726, Botrytis cinerea and strawberry. *Fems Microbiology Letters*, 285(1), pp 101–110.

Mamarabadi, M., Jensen, D. F. & Lübeck, M. (2009). An N-acetyl-beta-D-glucosaminidase gene, cr-nag1, from the biocontrol agent Clonostachys rosea is up-regulated in antagonistic interactions with Fusarium culmorum. *Mycological Research*, 113(Pt 1), pp 33–43.

Pal, K. K. & McSpadden Gardener, B. (2006). Biological Control of Plant Pathogens. *The Plant Health Instructor* [online],. Available from: http://www.apsnet.org/edcenter/advanced/topics/Pages/BiologicalControl.aspx. [Accessed 2017-04-23].

Pihet, M., Vandeputte, P., Tronchin, G., Renier, G., Saulnier, P., Georgeault, S., Mallet, R., Chabasse, D., Symoens, F. & Bouchara, J.-P. (2009). Melanin is an essential component for the integrity of the cell wall of Aspergillus fumigatus conidia. *BMC Microbiology*, 9, p 177.

Ravnskov, S., Jensen, B., Krudsen, I. M. B., Bodker, L., Jensen, D. F., Karlinski, L. & Larsen, J. (2006). Soil inoculation with the biocontrol agent Clonostachys rosea and the mycorrhizal fungus Glomus intraradices results in mutual inhibition, plant growth promotion and alteration of soil microbial communities. *Soil biology & biochemistry* [online],. Available from: http://agris.fao.org/agris-search/search.do;jsessionid=973CCD8F1FCA0133C62293DB04C42D23?request_locale=fr&recordID=US201301113042&query=&sourceQuery=&sortField=&sortOrder=&agrovocString=&advQuery=&centerString=&enableField=. [Accessed 2017-04-17].

Roberti, R., Veronesi, A., Cesari, A., Cascone, A., Di Berardino, I., Bertini, L. & Caruso, C. (2008). Induction of PR proteins and resistance by the biocontrol agent Clonostachys

rosea in wheat plants infected with Fusarium culmorum. *Plant Science*, 175(3), pp 339–347.

Schindler, D. & Nowrousian, M. (2014). The polyketide synthase gene pks4 is essential for sexual development and regulates fruiting body morphology in Sordaria macrospora. *Fungal genetics and biology: FG & B*, 68, pp 48–59.

Schroers, H.-J., Samuels, G. J., Seifert, K. A. & Gams, W. (1999). Classification of the Mycoparasite Gliocladium roseum in Clonostachys as C. rosea, Its Relationship to Bionectria ochroleuca, and Notes on Other Gliocladium-like Fungi. *Mycologia*, 91(2), pp 365–385.

Schultz, J., Milpetz, F., Bork, P. & Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences of the United States of America*, 95(11), pp 5857–5864.

Studt, L., Wiemann, P., Kleigrewe, K., Humpf, H.-U. & Tudzynski, B. (2012). Biosynthesis of Fusarubins Accounts for Pigmentation of Fusarium fujikuroi Perithecia. *Applied and Environmental Microbiology*, 78(12), pp 4468–4480.

Sun, Z.-B., Sun, M.-H. & Li, S.-D. (2015). Identification of mycoparasitism-related genes in Clonostachys rosea 67-1 active against Sclerotinia sclerotiorum. *Scientific Reports*, 5, p 18169.

Timsina, B. A. (2012). Evolution and Expression of polyketide synthase gene in the lichen-forming fungal families Cladoniaceae and Ramalinaceae. [online],. Available from: http://mspace.lib.umanitoba.ca/xmlui/handle/1993/23271. [Accessed 2017-04-17].

Toledo, A. V., Virla, E., Humber, R. A., Paradell, S. L. & Lastra, C. C. L. (2006). First record of Clonostachys rosea (Ascomycota: Hypocreales) as an entomopathogenic fungus of Oncometopia tucumana and Sonesimia grossa (Hemiptera: Cicadellidae) in Argentina. *Journal of Invertebrate Pathology*, 92(1), pp 7–10.

Utermark, J. & Karlovsky, P. (2008). Genetic transformation of filamentous fungi by Agrobacterium tumefaciens. *Protocol Exchange* [online],. Available from: http://www.nature.com/protocolexchange/protocols/427. [Accessed 2017-04-18].

Wang, X.-C., Zeng, Z.-Q. & Zhuang, W.-Y. (2017). The complete mitochondrial genome of the important mycoparasite Clonostachys rosea (Hypocreales, Ascomycota). *Mitochondrial DNA Part B*, 2(1), pp 180–181.

Watanabe, A. & Ebizuka, Y. (2004). Unprecedented Mechanism of Chain Length Determination in Fungal Aromatic Polyketide Synthases. *Chemistry & Biology*, 11(8), pp 1101–1106.

Weissman, K. J. & Leadlay, P. F. (2005). Combinatorial biosynthesis of reduced polyketides. *Nature Reviews Microbiology*, 3(12), pp 925–936.

Willems, E., Leyns, L. & Vandesompele, J. (2008). Standardization of real-time PCR gene expression data from independent biological replicates. *Analytical Biochemistry*, 379(1), pp 127–129.

Wright, P. J. & Pateman, J. A. (1970). Ultraviolet-light sensitive mutants of Aspergillus nidulans. *Mutation Research*, 9(6), pp 579–587.

Xu, R. & Li, Q. Q. (2008). Protocol: Streamline cloning of genes into binary vectors in Agrobacterium via the Gateway(R) TOPO vector system. *Plant Methods*, 4, p 4.

Xue, A. G. (2003). Efficacy of Clonostachys rosea strain ACM941 and fungicide seed treatments for controlling the root rot complex of field pea. *Canadian Journal of Plant Science*, 83(3), pp 519–524.

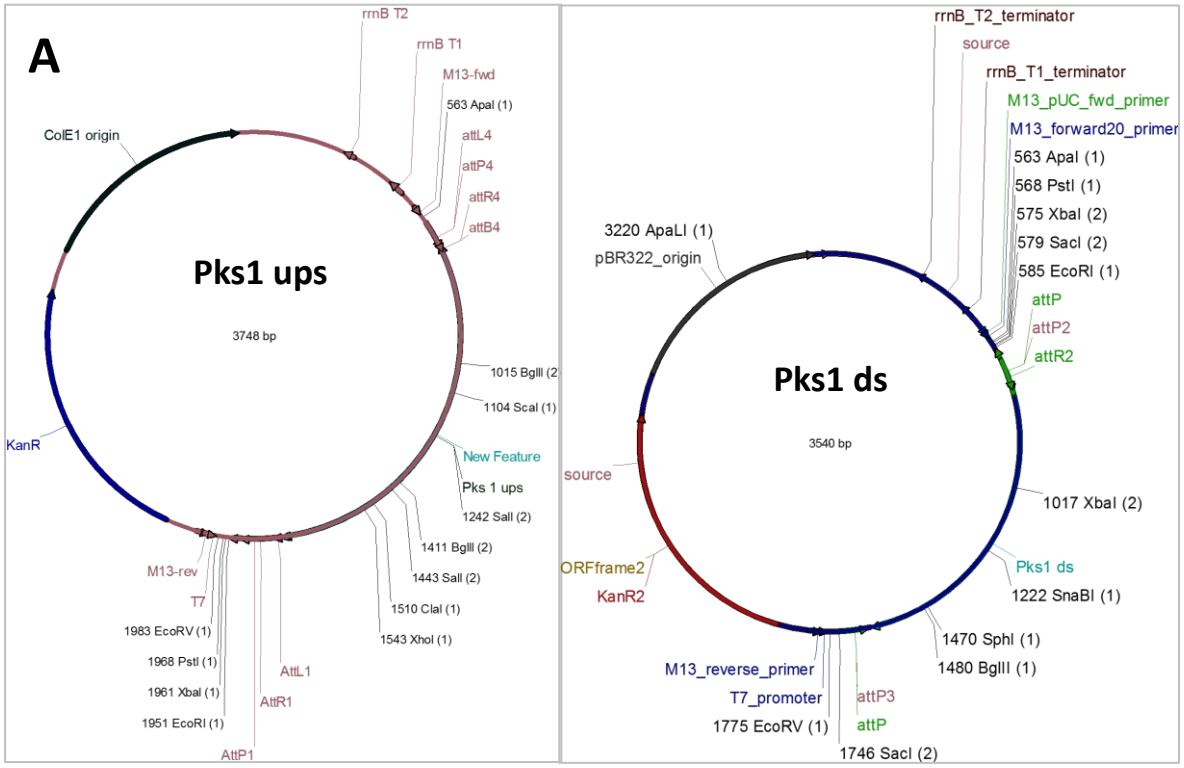
Yao, L., Tan, C., Song, J., Yang, Q., Yu, L. & Li, X. (2016). Isolation and expression of two polyketide synthase genes from Trichoderma harzianum 88 during mycoparasitism. *Brazilian Journal of Microbiology*, 47(2), pp 468–479.

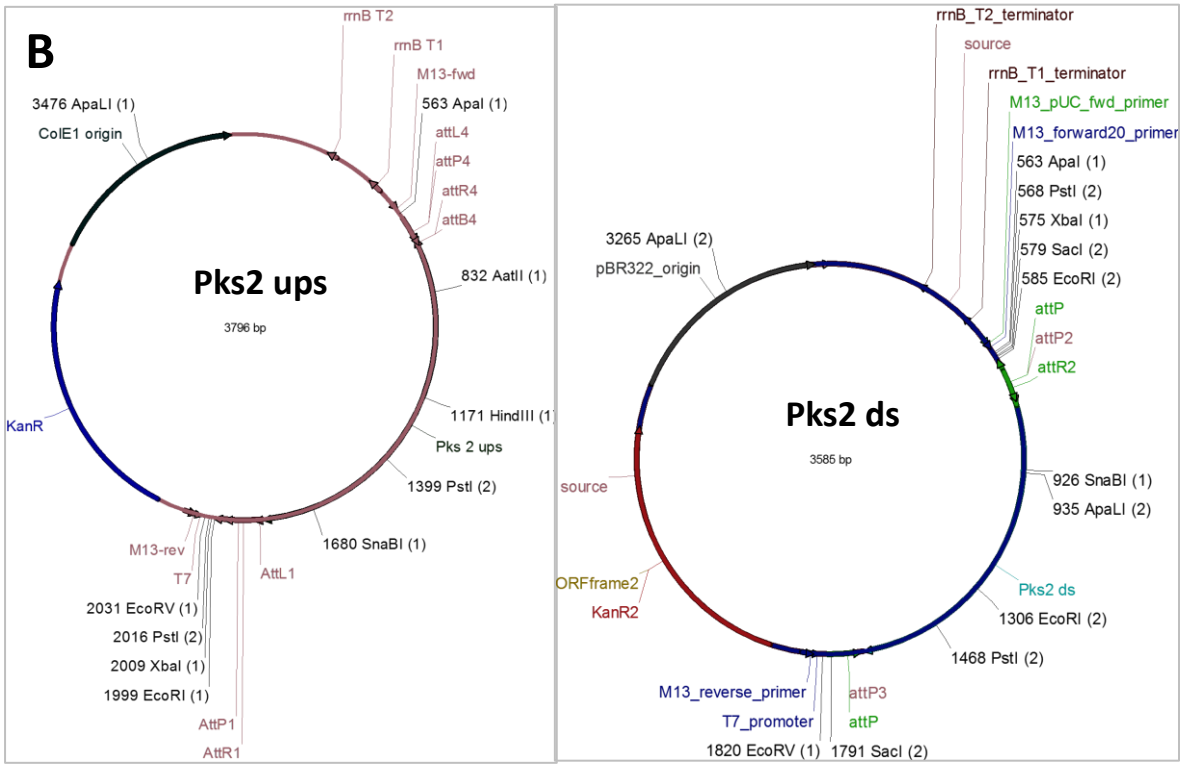
Zhai, M.-M., Qi, F.-M., Li, J., Jiang, C.-X., Hou, Y., Shi, Y.-P., Di, D.-L., Zhang, J.-W. & Wu, Q.-X. (2016). Isolation of Secondary Metabolites from the Soil-Derived Fungus Clonostachys rosea YRS-06, a Biological Control Agent, and Evaluation of Antibacterial Activity. *Journal of Agricultural and Food Chemistry*, 64(11), pp 2298–2306.

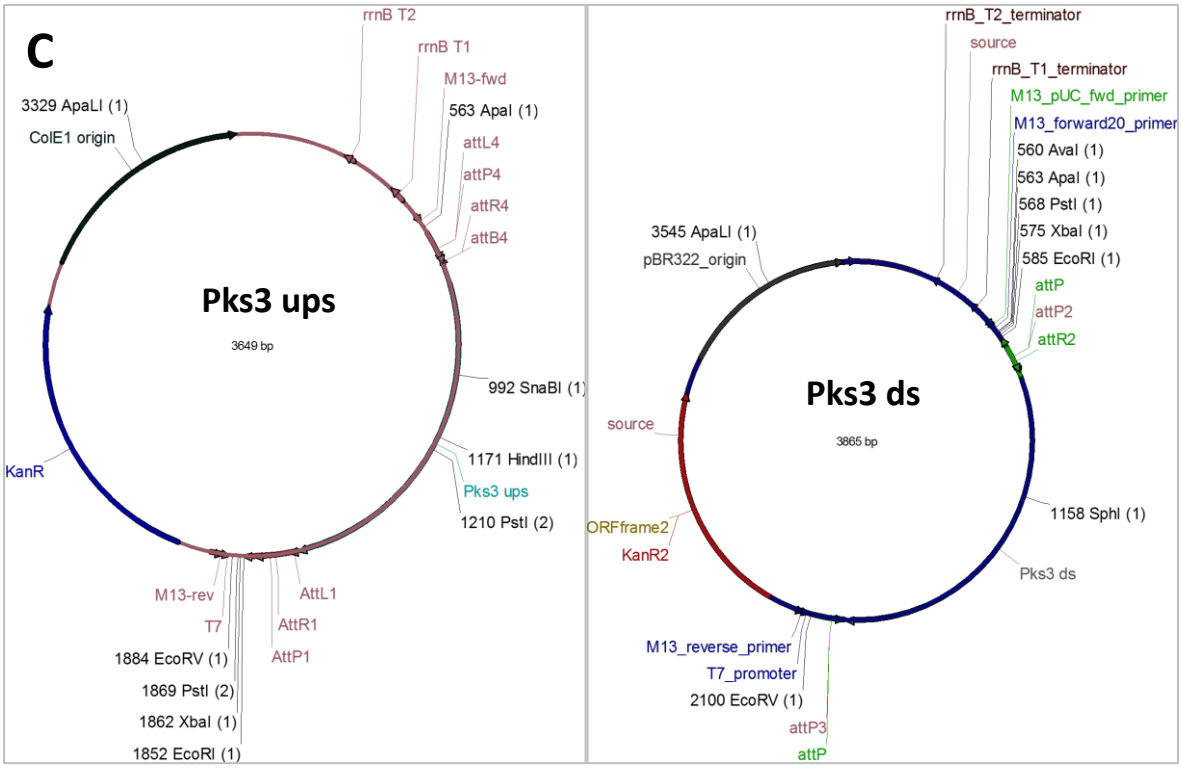
Zhou, R., Rasooly, R. & Linz, J. E. (2000). Isolation and analysis of fluP, a gene associated with hyphal growth and sporulation in Aspergillus parasiticus. *Molecular & general genetics: MGG*, 264(4), pp 514–520.

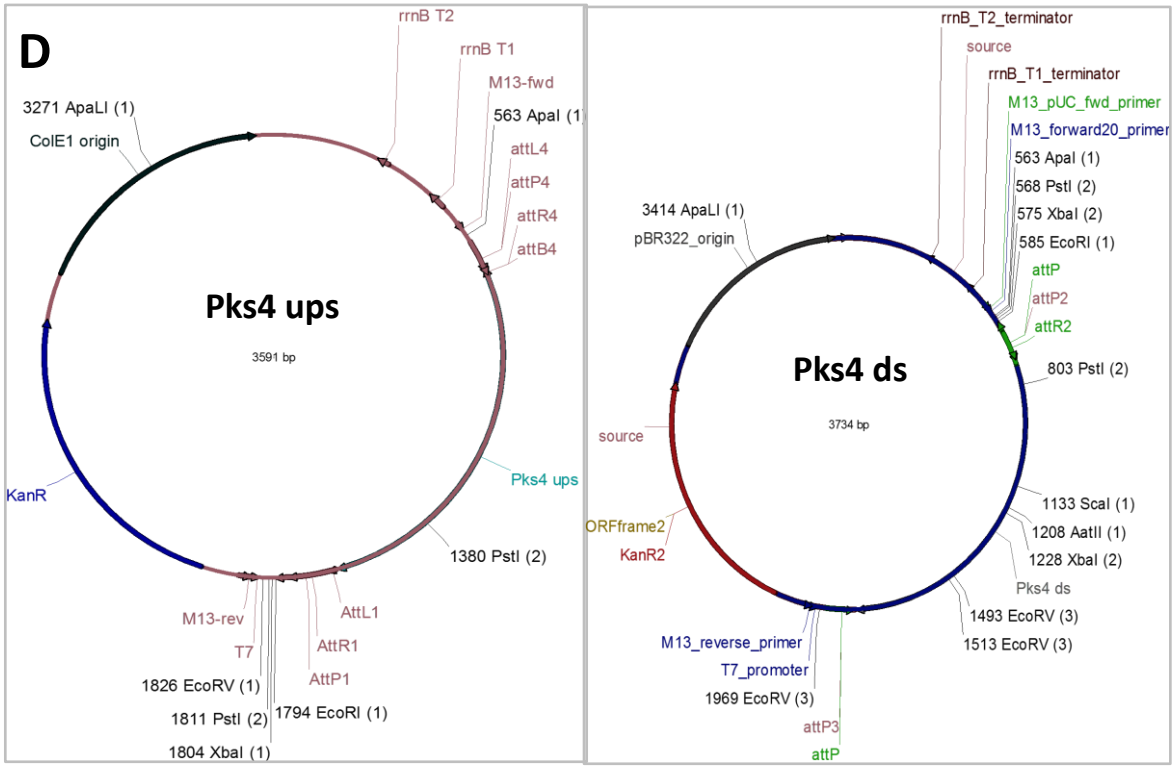
Zinedine, A., Soriano, J. M., Moltó, J. C. & Mañes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology*, 45(1), pp 1–18.

1 Supplementary information






C
Pks3 ups
3649 bp
rrnB T2
rrnB T1
M13-fwd
563 ApaI (1)
attL4
attP4
attR4
attB4
992 SnaBI (1)
1171 HindIII (1)
Pks3 ups
1210 PstI (2)
AttL1
AttR1
AttP1
1852 EcoRI (1)
1862 XbaI (1)
1869 PstI (2)
1884 EcoRV (1)
T7
M13-rev
KanR
3329 ApaLI (1)
ColE1 origin
Pks3 ds
3865 bp
rrnB_T2_terminator
source
rrnB_T1_terminator
M13_pUC_fwd_primer
M13_forward20_primer
560 AvaI (1)
563 ApaI (1)
568 PstI (1)
575 XbaI (1)
585 EcoRI (1)
attP
attP2
attR2
1158 SphI (1)
Pks3 ds
attP
attP3
2100 EcoRV (1)
T7_promoter
M13_reverse_primer
KanR2
ORFframe2
source
3545 ApaLI (1)
pBR322_origin


D
Pks4 ups
3591 bp
rrnB T2
rrnB T1
M13-fwd
563 ApaI (1)
attL4
attP4
attR4
attB4
Pks4 ups
1380 PstI (2)
AttL1
AttR1
AttP1
1794 EcoRI (1)
1804 XbaI (1)
1811 PstI (2)
1826 EcoRV (1)
T7
M13-rev
KanR
3271 ApaLI (1)
ColE1 origin
Pks4 ds
3734 bp
rrnB_T2_terminator
source
rrnB_T1_terminator
M13_pUC_fwd_primer
M13_forward20_primer
563 ApaI (1)
568 PstI (2)
575 XbaI (2)
585 EcoRI (1)
attP
attP2
attR2
803 PstI (2)
Pks4 ds
attP
attP3
1969 EcoRV (3)
T7_promoter
M13_reverse_primer
KanR2
ORFframe2
source
3414 ApaLI (1)
pBR322_origin
1133 ScaI (1)
1208 AatII (1)
1228 XbaI (2)
1493 EcoRV (3)
1513 EcoRV (3)

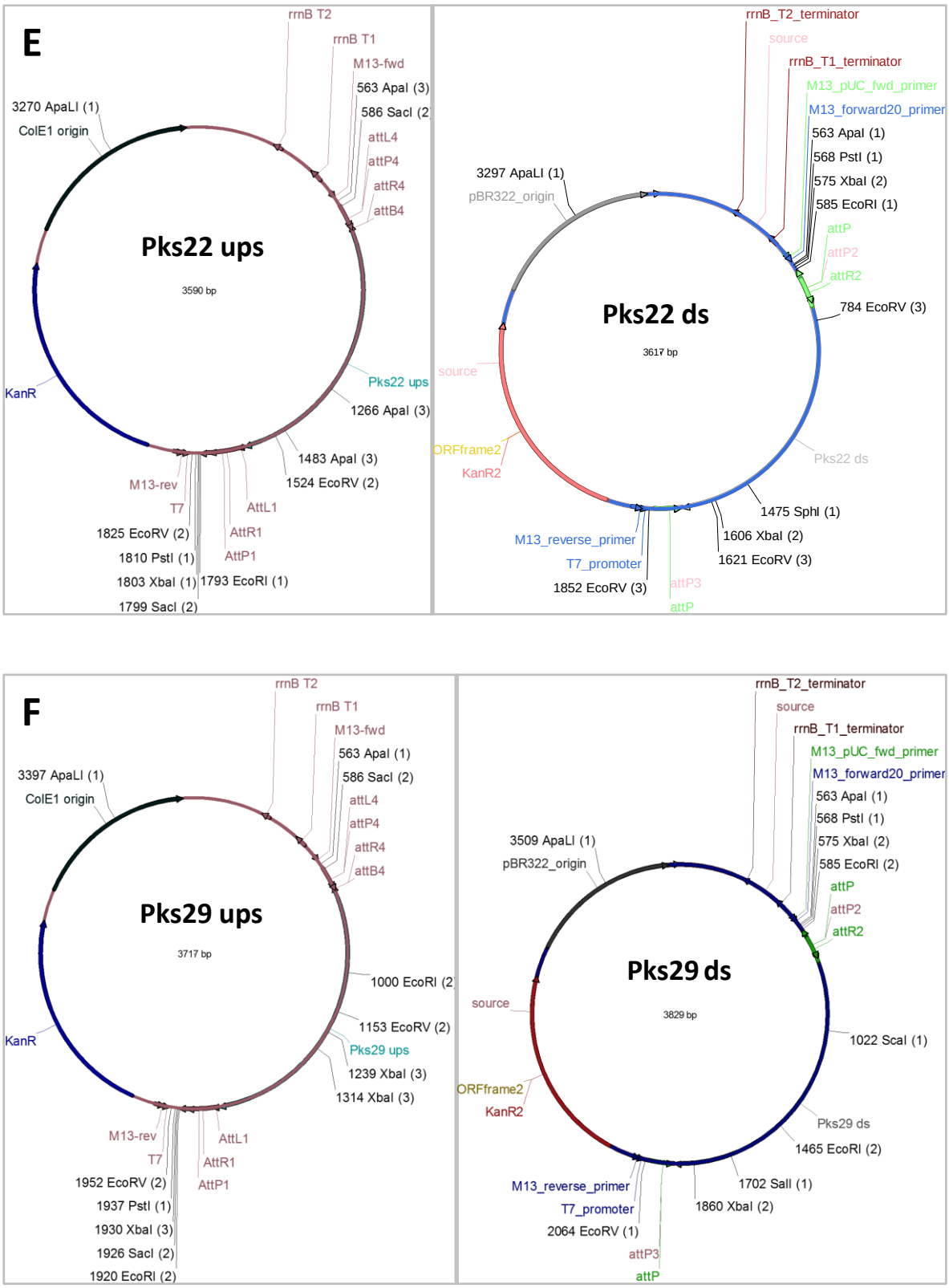


Figure S1. BP vector map with restriction enzyme to validate the correct insertion of the tansformants. BP vectors with restriction enzymes show the name and position of the restriction enzymes to validate the entry clones obtained from *pks1* ups/ds (A), *pks2* ups/ds (B), *pks3* ups/ds (C), *pks4* ups/ds (D), *pks22* ups/ds (E), and *pks29* ups/ds (F).

Table S1. *pks* expression analysis in *C. rosea* during pigmentation of *C. rosea* and during interactions of *C. rosea* with pathogenic fungus *F. graminearum* or *B. cinerea*

Gene name	Relative expression						
	Fungal-fungal interaction				Pigmentation		
	Cr-Cr	Cr-Bc	Cr-Fg	P-value	Control	Pigmentation	P-value
pks1	1.00^{AB}	3.64^{A}	0.44^{B}	0.024	1.00^{B}	933.17^{A}	0.001
pks2	0.78^{C}	1.31^{A}	0.98^{B}	0.001	1.00^{B}	646.27^{A}	0.001
pks3	0.55^{B}	3.43^{A}	0.53^{B}	0.013		**ND**	
pks4	0.59^{C}	1.89^{A}	0.90^{B}	0.00		**ND**	
pks5		**ND**				**ND**	
pks6	1.24^{A}	1.36^{A}	0.59^{A}	0.255	1.08^{B}	213.78^{A}	0.00
pks7	0.89^{A}	0.98^{A}	1.14^{A}	0.655	1.00^{B}	172.21^{A}	0.00
pks8	0.63^{B}	1.32^{A}	1.21^{A}	0.02	1.00^{B}	95.54^{A}	0.005
pks9	2.40^{A}	0.68^{B}	0.61^{B}	0.00	1.00^{B}	46.27^{A}	0.006
pks10	0.87^{AB}	2.28^{A}	0.50^{B}	0.036	1.00^{B}	47.37^{A}	0.005
pks11		**ND**				**ND**	
pks12	0.88^{B}	1.22^{A}	0.93^{B}	0.001	1.00^{B}	273.99^{A}	0.00
pks13	0.73^{B}	2.15^{A}	0.64^{B}	0.00	1.00^{B}	136.43^{A}	0.00
pks14	0.54^{B}	1.87^{A}	0.99^{AB}	0.049	1.00^{B}	184.06^{A}	0.003
pks15	0.78^{B}	1.59^{A}	0.81^{B}	0.002	1.00^{B}	131.42^{A}	0.00
pks16	0.48^{C}	2.08^{A}	1.00^{B}	0.002	1.00^{B}	244.72^{A}	0.00
pks17	0.65^{B}	3.29^{A}	0.47^{B}	0.016	1.00^{B}	464.65^{A}	0.001
pks18		**ND**				**ND**	
pks19	1.89^{A}	1.78^{A}	0.30^{B}	0.001		**ND**	
pks20	0.55^{B}	2.41^{A}	0.75^{B}	0.022	1.00^{B}	797.31^{A}	0.001
pks21	1.00^{A}	1.31^{A}	0.76^{A}	0.232	1.00^{B}	162.02^{A}	0.003
pks22	0.73^{C}	1.03^{B}	1.33^{A}	0.00	1.00^{A}	0.23^{B}	0.047
pks23	0.81^{A}	1.26^{A}	0.98^{A}	0.099	ND		
pks24	0.92^{A}	1.08^{A}	1.01^{A}	0.40	1.00^{B}	39.56^{A}	0.00
pks25		**ND**				**ND**	
pks26	0.96^{A}	1.30^{A}	0.80^{A}	0.086	1.00^{B}	13.95^{A}	0.011
pks27		**ND**				**ND**	
pks28	0.57^{C}	1.80^{A}	0.97^{B}	0.00	1.00^{B}	35.26^{A}	0.001
pks29	0.64^{B}	1.41^{A}	1.11^{A}	0.00	1.00^{A}	0.74^{A}	0.764
pks30	0.68^{AB}	3.75^{A}	0.39^{B}	0.05	1.00^{B}	20.48^{A}	0.00
pks31	1.02^{A}	0.86^{A}	1.14^{A}	0.528		**ND**	
pks32		**ND**				**ND**[1]	

[1] *pks*=Polyketide synthase encoding genes; Cr-Cr= *C. rosea* self-interaction; Cr-Bc= *C. rosea* and *B. cinerea* interaction; Cr-Fg=*C. rosea* and *F. graminearum* interaction; ND=Not detected. Superscript different letters indicate significant difference ($P \leq 0.05$) in gene expression among treatments.