A molecular analysis of the interaction between the biocontrol fungus *Clonostachys rosea* and the cereal pathogen *F. graminearum*

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Abstract

Modern agriculture is increasingly challenged by newly emerging crop diseases- many of them caused by fungal pathogens. At the same time, excessive application of chemical fungicides accelerates the development of resistant pathogen strains and may cause harmful effects on non-target organisms. Biological pathogen control based on living organisms is a promising component of more resilient disease management strategies, as it is thought to overcome some of the limitations of chemical control. However, for the development of more efficient biological control agents it will be crucial to gain a better understanding of the molecular interaction between the biocontrol organism and its prey. Here I studied the effect of three candidate genes on the antagonistic relationship between the biocontrol fungus *Clonostachys rosea* and the cereal pathogen *Fusarium graminearum.* The genes were selected based on their expression patterns during *C. rosea in- vitro* antagonism or *in-vivo* biocontrol and encode for the killer toxin-like chitinase CHIC1, the transcriptional regulator PRZ1 and a putative NLR-like receptor protein (NLRL). None of the candidate genes was differentially expressed in *C. rosea* cultures growing in dual culture with *F. graminearum*, however *nlrl* expression was increased in dual culture with *Botrytis cinerea*. *C. rosea* gene deletion strains were created through *Agrobacterium*- mediated transformation and screened for stress tolerance, *in- vitro* antagonism, and biocontrol efficiency for fusarium root rot disease. Growth rate analysis of *prz1* knock-out mutants showed decreased mycelial growth on agar plates supplemented with $CaCl_2$ or SDS. Furthermore, *F. graminearum* growth was increased in liquid cultures consisting of *C. rosea* Δ*chiC1* culture filtrates. Δ*nlrl* was the only gene deletion strain that affected *F. graminearum* growth in dual culture interaction, compared to wild type. Additionally, in this experiment, none of the created deletion strains performed differentially in biocontrol assays on wheat plants infected with *F. graminearum*. The data highlights differences in *C. rosea* gene regulation during *in- vitro* antagonism and biocontrol and indicates involvement of NLRL during *in- vitro* antagonism.

Keywords: Agrobacterium- mediated transformation, Biocontrol, *Clonostachys rosea*

Popular Science Summary

Like humans, plants can get diseased by microorganisms such as bacteria, fungi or viruses, that exploit the plants resources. Infections by microbial pathogens often cause symptoms such as wilting or root rot. In agricultural systems pathogen infections often occur much stronger than in nature, which leads to devastating crop losses. This is problematic because it affects the farmers' income but also contributes to food insecurities. Many agricultural methods exist to combat fungal plant pathogens in the field. One of them is the use of chemical fungicides that hinder reproduction or growth of the pathogen. Chemical fungicides can be very efficient, but if they are used in too large amounts there is a risk that individuals in a pathogen population develop resistance and are not affected by the fungicide anymore. One method that can be used instead of or together with fungicides is biological pathogen control. Here, living organisms are applied to the field that are natural antagonists of the pathogen, which means that it is harder for the pathogen to grow and reproduce when the biological control agent is present. One promising biological control agent is the fungus *Clonostachys rosea*, that helps to decrease disease symptoms of many fungal plant pathogens. For example, wheat seeds that are coated with spores of *C. rosea* are less susceptible to fusarium root rot disease caused by the fungus *Fusarium graminearum*. In this work I wanted to understand better how *C. rosea* can compete and antagonize *F. graminearum*. I therefore chose three *C. rosea* genes that have previously been shown to be important in the interaction with *F. graminearum* and generated knock-out mutants that are not carrying the specific gene anymore. If the gene would be important for the antagonism or biocontrol trait of *C. rosea*, the knock-out mutants would perform worse in phenotypic assays compared to the non-mutated *C. rosea* strain (wild type). The chosen genes were *chiC1*, which is involved in cell wall disruption of the prey; *prz1*, which mediates stress responses inside the *C. rosea* cell and *nlrl*, which is putatively involved in reception of the pathogen. I could show that PRZ1 is important for *C. rosea* growth during external abiotic stresses. NLRL further plays a role during interaction of *C. rosea* with different plant pathogens and NLRL knock-out mutants performed worse in antagonistic assays with *F. graminearum*. None of the selected genes seems to play a crucial role in the biocontrol trait of *C. rosea*.

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Abbreviations

ATMT	*Agrobacterium tumefaciens* mediated transformation
ATP-binding cassette transporters	ABC transporters
BCA	Biological control agent
CWDEs	Cell wall degrading enzymes
ds	Downstream
FHB	Fusarium head blight
FR	Flanking region
FRR	Fusarium root rot
GOI	*Gene of interest*
GWAS	Genome wide association studies
HR	Homologous recombination
Hyg	Hygromycin
MFS	Major facilitator superfamily
MoA	Mode of action
NLR	NOD-like receptor
RNA-seq	RNA sequencing
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
ups	Upstream
WT	Wild type

1. Introduction

1.1. *Clonostachys rosea* as a biological control agent of plant pathogens

Fungal plant pathogens are causing significant threats to agricultural systems by contributing with approx. 13 % to the worldwide annual losses in crop production (Moore et al., 2020). To control the severity of disease outbreaks and ensure a sufficient production of food, conventional farming systems often depend on the application of chemical fungicides that decrease pathogen growth or reproductive ability. Although the approval of active substances for fungicide use in the European Union (EU) is tightly regulated by EU laws (EC no. 1107/2009, 2009), fungicide application rates can easily exceed critical values as agricultural land use is intensifying. However, overcritical fungicide use can lead to the occurrence of resistant pathogen strains and may impose health risks for agricultural workers or consumers (Jensen et al., 2016). Alternative to chemical pathogen control, biological control (or biocontrol) is the *"exploitation of living agents to combat pestilential organisms (incl. pathogens […]) for diverse purposes to provide human benefits"* (Stenberg et al., 2021). The use of living organisms instead of chemical compounds reduces the risks of emerging fungicide resistances, imposes less harmful effects on environment and is compatible with integrated pest management practices and organic agriculture (Jensen et al., 2016).

One microbial organism for biological control is the mycoparasitic ascomycete fungus *Clonostachys rosea* (teleomorph name: *Bionectria ochroleuca*). Numerous *C. rosea* strains have been isolated from soil samples and plant roots worldwide (Broberg et al., 2018; Sun et al., 2020) that show great potential to control various economically important fungal plant pathogens including *Botrytis cinerea*, *Fusarium graminearum* and *Rhizoctonia solani* (Sun et al., 2020). Moreover, *C. rosea* has been shown to have a negative impact on plant parasitic nematodes (Iqbal et al., 2019) and members of the oomycota phylum (Xue, 2003), while showing positive effects on plant growth (Ravnskov et al., 2006). The high number of prey species and wide distribution of strains across different climates are making *C. rosea* a promising organism for the commercial development of biological control

agents (BCAs). Certain *C. rosea* strains have been patented for use against crop diseases (Demissie et al., 2020). During a Nordic program to identify new microbial antagonists of seed borne diseases in cereals (Knudsen et al., 1997), the *C. rosea* strain IK726 was isolated from barley roots in Denmark and subsequently reported to be a strong BCA of cereal pathogens of the Fusarium genus (Jensen et al., 2000) and numerous other plant pathogens (Karlsson et al., 2015). In general, *C. rosea* IK726 has a strong rhizosphere-competence through fast mycelial growth- and colonization rates (Broberg et al., 2018; Jensen et al., 2000).

Fusarium graminearum (teleomorph name: *Gibberella zeae*) is the main causal agent of fusarium head blight (FHB) and fusarium root rot (FRR) diseases and imposes an increasing risk on northern Europe's production of small grain cereals (Nielsen et al., 2011). Infected seeds are characterized by low germination rates and poor tiller production, while diseased plants show reddish-brown discoloration at the base of the stem and lower grain yield. In addition to devastating losses on the field, *F. graminearum* produces mycotoxins that accumulate in the grain during infection, leading to significant post-harvest losses (Kosawang et al., 2014; Nielsen et al., 2011). Due to similar geographic niches and reported antagonism of *C. rosea* IK726 towards *F. graminearum*, IK726 is an attractive candidate strain for the commercialization of BCAs against FHB and FRR. However, for such a development, it is important to gain a better understanding of the mechanisms underlying this control.

1.2. The molecular interaction of *C. rosea* and *F. graminearum*

Microbial biological control is a complex trait. Increasing experimental evidence is demonstrating that successful BCAs often have a strong antagonistic nature through secretion of enzymes and secondary metabolites that are directly involved in mycoparasitism. Secondly, many BCAs can tolerate toxic compounds, enabling continued growth and competition in the presence of the prey (Sun et al., 2020). During the last years, the genomes of several *C. rosea* strains, including IK726, have been sequenced (Broberg et al., 2018; Karlsson et al., 2015), facilitating a molecular understanding of *C. rosea* biological control against *F. graminearum* and other plant pathogens.

1.2.1. Secondary metabolites and cell wall degrading enzymes

One important aspect of necrotrophic mycoparasitism is the secretion of antifungal proteins and secondary metabolites. Analysis of the *C. rosea* IK726 genome, for instance, revealed a high number of polyketide synthase (*pks*) genes (31 genes)

involved in the production of polyketide secondary metabolites (Fatema et al., 2018). Polyketides are a functionally diverse group produced by bacteria, fungi and plants, that may exhibit antifungal properties. Deletion of selected *pks* genes in *C. rosea* IK726 revealed a reduced ability to antagonize *B. cinerea* and *F. graminearum* on growth medium and barley plants, suggesting a role in biocontrol. However, absent phenotypes in other *pks* deletion mutants also indicate functional redundancy in that gene family and pinpoint possible limitations in phenotypic assays (Fatema et al., 2018). Fungi-fungi interactions are additionally shaped by the secretion of proteins involved in recognition and defence. Lysin motif containing proteins (LYSM), for example, contain carbohydrate-binding domains and can function both as hydrolytic enzymes and receptor molecules (Buist et al., 2008). Filamentous fungi such as *F. graminearum* and *Trichoderma atroviride* often contain a high number of *lysm* genes (13 and 12, respectively), but surprisingly, the analysis of the *C. rosea* IK726 genome revealed only three *lysm* genes (Dubey et al., 2020). The deletion of two *C. rosea lysm* genes, resulted in a reduced biocontrol effect of *B. cinerea* and *F. graminearum in-planta*, while knock-out mutants of the third *lysm* gene showed no differences in biocontrol ability of *B. cinerea* compared to wild type (WT; Dubey et al., 2020). Functional characterization identified the latter gene as the killer toxin-like chitinase gene *chiC2*, described by Tzelepis et al. (2015).

Secretion of cell wall degrading enzymes (CWDEs) is another important factor of *C. rosea* mycoparasitism. Fungal cell walls (CWs) consist mainly of glycoproteins and polysaccharides, such as chitin and glucan (Bowman & Free, 2006). The production of proteases, chitinases and glucanases, is therefore enabling mycoparasites to degrade the prey's cell wall and facilitate necrotrophism (Demissie et al., 2020). Fungal chitinases belong to glycoside hydrolases (GH) family 18 and 14 GH18 genes were identified in the genome of *C. rosea* IK726. Gene expression analysis using revere transcription quantitative PCR (RT-qPCR) showed an increased expression of two GH18 genes when *C. rosea* was grown in liquid medium with homogenized mycelium of *R. solani* and *B. cinerea*. Growth on *F. graminearum* mycelium did not induce the expression of any chitinase gene (Tzelepis et al., 2015). However, transcriptome analysis of *C. rosea* IK726 in dual culture confrontation assays, revealed a 50-fold increased expression of the killer toxin-like chitinase *chiC1* (CRV2T00011101; table 1) during interaction with *F. graminearum* (Nygren et al., 2018), suggesting a role of that gene during *in-vitro* antagonism. Similar mixed results during characterization of CWDEs in *C. rosea* antagonism have been reported (Demissie et al., 2020) and indicate a strong influence of the experimental approach on the obtained results. For functional characterization of CWDEs involved in biocontrol, it is additionally important to consider that cell wall composition of phytopathogens from different phyla can

differ, for example between fungal and oomycete pathogens (Inglis & Kawchuk, 2002).

1.2.2. Tolerance of mycotoxins

Mycoparasitic fungi require complex strategies to cope with toxic compounds produced by their prey. In *C. rosea* IK726, tolerance mechanisms involve detoxification of the antifungal *F. graminearum* mycotoxin zearalenone (ZEA) by the enzyme zearalenone hydrolase (ZHD101), as well as active efflux of the mycotoxin from the cell through various membrane transporters (Kosawang et al., 2014). One transporter family mediating tolerance towards *F. graminearum* mycotoxins is the ATP-binding cassette (ABC) transporter family. Constituting one of the largest protein families, ABC transporters facilitate ATP-dependent efflux of a broad range of endogenous and exogenous toxic compounds (Kovalchuk & Driessen, 2010). Genome analysis of the *C. rosea* IK276 genome revealed a high number of *abc* genes (86 genes) compared to related mycotoxin-producing and mycoparasitic fungal species (Karlsson et al., 2015). *C. rosea* IK726 *abc* deletion mutants showed reduced growth rate on medium supplemented with ZEA and certain fungicides, indicating a profound, yet specific, effect of ABC transporters. Reduced tolerance of *F. graminearum* mycotoxins also decreased the biocontrol ability of *C. rosea* IK726, as barley seedlings from seeds coated with spores of *abc* deletion strains developed more severe symptoms of FRR in biocontrol experiments compared to seedlings from seeds treated with the WT (Dubey et al., 2014; 2016). Similar to the ABC transporter family, the *C. rosea* IK726 genomes involves a large number of genes (620 genes) encoding for transporters of the major facilitator superfamily (MFS) (Karlsson et al., 2015). Both transporter families play crucial roles in secreting toxic compound from the cell into the outer environment, but unlike ABC transporters, efflux through MFS transporters is driven by a proton gradient over the membrane that must be established prior to MFS efflux (Roohparvar et al., 2007). Moreover, MFS transporters show less conserved secondary and tertiary structures, suggesting a division into several hundred subfamilies based on sequence similarity and transported substrates (Nygren et al., 2018). Using an RNA-sequencing (RNA-seq) approach, Nygren at al. (2018) identified that 53 % (22) of differently regulated genes during dual culture interaction of *C. rosea* IK726 with *F. graminearum* and *B. cinerea*, respectively, encoded for MFS transporters. Interestingly, only three *mfs* transporter genes were induced during *C. rosea* interaction with both pathogens, while the response of the other *mfs* genes was specific towards one pathogen. Although the expression of two *mfs* genes was highly induced during dual culture interaction with *F. graminearum* (700-fold and 800-fold, respectively), *mfs* deletion mutants did not show phenotypic effects during dual culture interaction or biocontrol assays of FRR (Nygren et al., 2018). Given the high number of *mfs* paralogs in *C. rosea*, it is

plausible that other MFS transporters may compensate for the deleted genes and mask the phenotypic effects through functional replacement. However, absent phenotypes during biocontrol assays may also suggest differences in gene regulation during *in- vitro* antagonism and *in- vivo* biocontrol. Using a genome-wide association approach (GWAS), Dubey et al. (unpublished) screened a collection of whole-genome sequenced *C. rosea* strains to identify genes contributing to high performance during biocontrol assays of FRR on wheat plants. One identified gene encodes for an MFS transporter, putatively belonging to the 2.A.1.3.73 family. For simplicity, this gene will be referred to as *mfs* in the following text (Saier et al., 2015; CRV2T00015724; table 1). The 2.A.1.3.73 transporter family comprises of antiporters functioning in drug: H^+ efflux and the *Mycosphaerella graminicola* homolog MgMfs1 showed a role in mediating resistance towards fungicides and mycotoxins (Roohparvar et al., 2007).

1.2.3. Pathogen recognition and stress responses

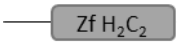
Early recognition of the prey and initiation of an immune response are essential factors for successful mycoparasitism. Like plants and animals, fungi possess an innate immune system involving NOD-LIKE RECEPTOR (NLR)-LIKE proteins (Loquet & Saupe, 2017). Analysis of approx. 200 fungal genomes revealed a high number of putative NLR-LIKE receptors (5600 genes) with high structural diversity (Dyrka et al., 2014). Similar to NLRs, all identified NLR-LIKE proteins (NLRLs) show a tripartite domain structure with a central nucleotide binding (NB) domain, an effector domain and a ligand-binding domain (Dyrka et al., 2014; Loquet & Saupe, 2017). However, fungal NLRLs display a higher structural variation, particularly within the N-terminal effector domain, indicating an involvement in diverse cellular functions. The well-studied heterokaryon incompatibility (HET) domain, for instance, induces the production of pore-forming toxins in filamentous ascomycetes when genetically different HET loci are received. This form of programmed cell death, termed heterokaryon incompatibility (HI), prevents genetic exchange with incompatible individuals (Abrams et al., 2005), but also triggers fungal immune response against pathogenic viruses and bacteria (Uehling et al., 2017). Other domains of NLRLs, such as the nucleoside phosphorylase PFS domain, play regulatory roles and homologous PFS containing proteins in bacteria and animals have signalling roles, triggering gene expression and cell proliferation (Abrams et al., 2005). Using a GWAS approach, Dubey et al. (unpublished) identified a putative NLRL protein to be involved in *C. rosea* biocontrol against FRR. In this text, this gene will be referred to as *nlrl* (CRV2T00017633; table 1). The identified protein has a tripartite domain structure composed of a N-terminal nucleoside phosphorylase (NP) domain, a central nucleotide binding (NB) domain and a C-terminal tetratricopeptide repeat (TPR) domain (Marchler-Bauer et al., 2015). NLRL proteins with similar domain compositions were previously described

to function as protein binding and inducers of signalling cascades (Abrams et al., 2005). However, little is known about specific NLRL responses in *C. rosea*, as well as their role in mycoparasitism and biocontrol.

Molecular responses during fungal-fungal interactions are initiated at the receptive site and transduced via signalling pathways. A well-known component of signal transduction in both prokaryotes and eukaryotes is the concentration of intracellular free calcium (Ca^{2+}). The fungal cytosolic Ca^{2+} concentration is approx. 100 nM in resting state and therefore more than 10000-fold lower that in the cell surroundings (Roy et al., 2020). When external stresses are encountered, Ca^{2+} channels in the plasma membrane open and the cytosolic Ca^{2+} concentration raises, modulating a wide range of cellular processes, including growth patterns and gene expression (Liu et al., 2015). However, highly increased intracellular Ca^{2+} concentrations are toxic for fungal cells and Ca^{2+} homeostasis must be restored quickly after stress responses have been performed. In the calcium-calcineurin signalling pathway, calcineurin proteins bind cytosolic Ca^{2+} and activates several transcription factors such as the calcineurin responsive zinc finger 1 (CRZ1) and its homolog PRZ1. CRZ1/PRZ1, in turn, induces the biosynthesis of Ca^{2+} pumps located in the membrane of the vacuole and Golgi apparatus. Subsequently, cytosolic Ca^{2+} concentrations are reduced due to intake by the cell organelles (Liu et al., 2015). *Pmc1* encodes for a Ca^{2+} pump controlled by PRZ1 and is involved in the intake of Ca^{2+} into the vacuole. *Pmc1* deletion strains of *Saccharomyces cerevisiae* and *Candida albicans* showed reduced growth rates in the presence of high Ca^{2+} as well as sensitivity towards other ionic and osmotic stresses (Jia et al., 2018). During a project aiming to identify small RNAs involved in the plant growth promoting ability of *C. rosea*, Piombo et al. (unpublished) showed a role of *C. rosea prz1* (CRV2T00002266; table 1), suggesting a putative involvement in biocontrol.

Table 1. C. rosea candidate genes selected for creation of knock-out mutants and functional characterization.

Protein ID	Annotation	Putative functional domains	Basis of selection
CRV2T00017633	NLR-like receptor		Dubey et al., unpublished
CRV2T00015724	MFS transporter, family 2.A.1.3.73		Dubey et al., unpublished
CRV2T00011101	Killer toxin-like chitinase CHIC1		Nygren et al., 2018

CRV2T00002266	Transcriptional regulator PRZ1		100 aa	Piombo et al., unpublished

Candidate genes were selected from the listed publications and protein sequences and domains were annotated using the NCBI conserved domain database and the InterPro protein classification database. The bar indicates a protein sequence of 100 aa length. NLR, NOD-like receptor; NP, nucleoside phosphorylase; NB, nucleotide binding; TPR, tetratricopeptide repeat; MFS, major facilitator superfamily; GH, glycoside hydrolase; Zf, zinc finger.

1.3. Objective

The aim of this thesis was to functionally characterize the previously described NLR-like receptor (Dubey et al., unpublished), MFS transporter (Dubey et al., unpublished), CHIC1 chitinase (Nygren et al., 2018) and PRZ1 transcription factor (Piombo et al., unpublished); and evaluate their role in the molecular interaction of *C. rosea* IK726 and *F. graminearum* PH-1. For that, gene expression patterns of the candidate genes in dual culture interaction were studied and knock-out mutants of the candidate genes were created using an *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol. *C. rosea* mutants were evaluated in their ability to control FRR using a bioassay on wheat plants. The performance of the mutants for *in- vitro* antagonism of *F. graminearum*, *B. cinerea* and *R. solani* was analysed during dual culture interactions on agar plates, as well as the growth rate under different stress conditions. In a separate dual culture interaction assay, 53 *C. rosea* strains were screened for their ability to antagonize and overgrow *F. graminearum*. In the future the generated dataset could be used for identifying involved genes using GWAS analysis.

Creation of *C. rosea* deletion strains was successful for *chiC1*, *prz1* and *nlrl*, but not for *mfs*. Δ*chiC1* mutants were not impaired in nutrient acquisition, but *F. graminearum* growth rates were affected by Δ*chiC1* culture filtrates. Δ*prz1* showed reduced tolerance to ionic stress and cell wall disruptions. NLRL appears to be involved in *in- vitro* antagonism, as indicated by both gene expression analysis and dual culture interaction studies. Neither candidate gene showed to play an essential role during *in- vivo* biocontrol of FRR in wheat plants.

2. Materials and Methods

2.1. General conditions for cell cultures and molecular methods

Strains and Culture conditions

Clonostachys rosea strain IK726, *F. graminearum* strain PH-1, *B. cinerea* strain B05.10 and *R. solani* strain SA1 were maintained on potato dextrose agar (PDA; Sigma-Aldrich) in 25°C, unless otherwise specified. *C. rosea* liquid cultures were grown in ½ strength potato dextrose broth (PDB; Sigma-Aldrich) in 25 °C, on a rotary shaker at 130 rpm.

Nucleic acid extractions and cDNA synthesis

For DNA extractions, agar plugs with growing *C. rosea* mycelium were inoculated in Erlenmeyer flasks containing approx. 10 ml of liquid ½ strength PDB medium and incubated in 25°C in darkness for 3 days. 100-200 mg of freshly grown mycelium were homogenized with cetrimonium bromide (CTAB) using glass beads and homogenizer (5000 rpm 2x 30 sec with 15 sec interval) as described by Nygren et al. (2008). Homogenized samples were incubated for 1-2 hours at 65°C and 600 µl chloroform was added. After centrifugation, 1000 µl isopropanol was added to the upper phase and the samples were incubated for 30 min at -20°C. Precipitated DNA was collected by centrifugation, washed with 70 % ethanol, and suspended in 50 µl milliQ H_2O. DNA concentrations were measured using a NanoDrop® spectrometer.

Total RNA was extracted from *C. rosea* mycelium using the RNeasy Plant mini kit (Qiagen) according to the manufacturer's instructions. RNA concentrations and purity ratios (230/260; 280/260) were measured using NanoDrop® spectrometer. Genomic DNA contamination was removed by DNaseI treatment of 1 µg RNA following the instruction provided by Sigma-Aldrich. For cDNA synthesis, DNase-treated RNA was reverse transcribed following the instructions of the iScript cDNA synthesis kit (Bio-Rad).

General PCR and gel electrophoresis settings

Primers used for PCR were designed using the DNAStar Seqbuilder® software. Unless otherwise specified, PCR reactions were carried out in 25 µl reaction volume, according to the DreamTaq® polymerase user guide (Thermo Fisher). In standard PCR reactions, variable thermal cycling conditions were chosen as 60°C annealing temperature and 32 cycles. PCR products were loaded on a 1 % agarose gel supplemented with 1 µl Nancy-520 (Sigma-Aldrich) per 50 ml agarose gel and DNA bands were separated by applying an electrical current of 120-150 V. All PCR primers with respective annealing temperatures and elongation times are listed in table S1.

2.2. Creation and validation of *C. rosea* deletion strains

Construction of gene deletion cassette

Expression vectors carrying the *hygB* gene, conferring resistance to Hygromycin (Hyg), were created using the MultiSite Gateway® technology. Gateway specific PCR primers were designed to amplify 600-1000 bp of 3' and 5' flanking regions (FRs) of the selected candidate genes (Table 1) from *C. rosea* WT genomic DNA. Amplified FRs were purified from a 0.8 % agarose gel using the Gene JET gel extraction kit® (Thermo Scientific) and cloned into Gateway specific pDONR™ P1-P4 and pDONR™ P3-P2 vectors by Gateway BP recombination reactions following the MultiSite Gateway® protocol. Four µl of BP reaction mix were used for transformation of competent TOP10 *E. coli* cells using a heat shock at for 30 sec at 42°C. Positive transformants were selected on lysogeny broth (LB) agar plates containing 50 µg/ml Kanamycin (Kan) and individual colonies were inoculated in 5 ml liquid LB overnight cultures at 37°C. Plasmid DNA was extracted using the Gene JET plasmid miniprep kit® (Thermo Scientific) and the expression of correct plasmids was confirmed by enzymatic digestion (37°C, 25 min) and gel electrophoresis.

Three independent entry clones carrying the 3' FR, 5' FR, and Hyg resistance cassette (provided by Mukesh Dubey, Department of Forest Mycology and Plant Pathology), respectively, were used for Gateway LR reaction and cloned into destination vector pPM43GW (Karimi et al., 2005). Four µl LR reaction were used for transformation of TOP10 cells and positive transformants were selected on LB agar plates containing 50 µg/ml Spectinomycin (Spec). Plasmid DNA was extracted as described above and positive expression vectors (Figure S1) were used for transformation of *Ag. tumefaciens* strain AGL-1.

Transformation of *Ag. tumefaciens*

Competent *Ag. tumefaciens* cells were prepared as described elsewhere (Xu & Li, 2008). A colony of *Ag. tumefaciens* was inoculated in 50 ml liquid LB medium and incubated until OD_{600}= 0.5. *Ag. tumefaciens* cells were harvested by centrifugation and rinsed with 10 ml of 20 mM ice- cold $CaCl_2$. Centrifugation was repeated and the cell pellet suspended in 1 ml of 20 mM $CaCl_2$. Aliquots of 0.1 ml were immediately frozen in liquid nitrogen and stored in -80°C.

For *Ag. tumefaciens* transformation, 1 µg purified DNA of the expression vector was added to freshly thawn *Ag. tumefaciens* cells and heat shock was performed for 5 min at 37°C. Following the protocol described by Xu & Li (2008), 150 µl LB medium was added and transformed *Ag. tumefaciens* cells recovered through incubation at 28°C for 3 h. *Ag. tumefaciens* cells were spread on LB plates containing 50 µg/ml Spec and 50 µg/ml Rifampicin (Xu & Li, 2008) and positive colonies formed after 3 d incubation at 28°C. Transformed colonies were transferred to liquid LB medium containing 50 µg/ml Spec and 50 µg/ml Rif and the grown cell culture was stored in -80°C in an 80 % glycerol stock.

Agrobacterium tumefaciens - mediated transformation of *C. rosea*

For *C. rosea* transformation, induction medium (IM) and GM7 medium were prepared as described elsewhere (Utermak & Karlovski, 2008). Individual transformed *Ag. tumefaciens* colonies were used for inoculation of 10 ml liquid LB medium and incubated at 28°C until OD_{600}= 0.5-0.9. The bacterial culture was harvested by centrifugation and the pellet was washed twice with liquid IM. The washed cell pellet was re-suspended in liquid IM, the cell density was adjusted to OD_{600}= 0.15-0.2 and Acetosyringone (AS) was added to a final concentration of 200 µM. The bacterial culture was incubated until OD_{600}= 0.3-0.4 and mixed in equal parts with freshly harvested *C. rosea* conidia with a spore density of 10^7 spores/ml. Two hundred µl of the mixtures was spread on a sterilized cellophane sheet placed in IM agar plates supplemented with 200 µM AS. The plates were incubated at 24°C for 55-60 h before the cellophane membrane was transferred to GM7 agar plates containing 400-500 µM Cefotaxime (Cef) and 200 µg/ml Hygromycin. The plates were incubated at 25°C for 3-5 d before positive *C. rosea* transformants became visible.

Validation of gene deletion strains

To confirm correct integration of *hygB* into the *C. rosea* genome, genomic DNA was extracted from colonies growing on Hyg selection plates using the CTAB extraction method as described above. PCR was performed with primers located approx. 100 bp upstream or downstream of the targeted integration side and within

the *hygB* gene (Table S1). *C. rosea* mutants with correct integration of *hygB* were sub-cultured on PDA plates without selection pressure four times and finally transferred to PDA plates containing 200 µg/ml Hyg for determining the stability of transformants. Conidia of the mutants were harvested and spread on a fresh PDA plate for single spore purification. Growing colonies were transferred to PDA plates containing 200 µg/ml Hyg. To confirm the complete deletion of the gene of interest (GOI), putative *chiC1*, *prz1* and *nlrl* mutants were inoculated in SMS medium (Dubey et al., 2012) with 0,1 % glucose, SMS + 1 % glucose or PDB medium, respectively, and incubated for 3 d. RNA was extracted from mycelium and cDNA was synthesized as described above. WT and gene deletion strains were tested for gene expression by reverse transcription PCR (RT-PCR) using the primers designed for RT-qPCR (Table S1). For all phenotypic assays, three independent transformants of each mutant line were used.

2.3. Growth rate analysis

Osmotic and ionic stress inducers

For growth rate analysis, a 3 mm diameter agar plug from the growing mycelial front of *C. rosea* Δ*prz1* was inoculated on CZ agar plates supplemented with 400 mM $CaCl_2$, 0,025 % SDS, 1 M NaCl and 10 mM $LiCl_2$, respectively (Nygren et al., 2018).

Variation of carbon sources

Growth rates of *C. rosea* Δ*chiC1* was analyzed on SMS agar plates, supplemented with 1 % (w/v) glucose, 0.5 % (w/v) colloidal chitin (Sigma-Aldrich) or 0.9 % (w/v) of *F. graminearum* cell wall components as sole carbon source. Cell wall material was isolated following a modified protocol by Inglis et al., (2002). *F. graminearum* was incubated in 25°C in darkness, for 7 d and hyphae were harvested through filter paper. Hyphae were washed with sterile H_2O, grinded to a fine powder in liquid nitrogen and suspended in dH_2O containing 0.1 % SDS. Cell wall components were collected through centrifugation and the pellet was washed 6 times with sterile H_2O. The pellet was dried at 37°C o/n and stored at -20°C. Growth rates were measured daily at 25°C in darkness in triplicates.

2.4. Antagonism and biocontrol assays

Dual culture interactions on solid medium

A 7 mm-diameter agar plug from the growing edge of a *C. rosea* culture was inoculated on one side of Czapek dox agar plates (Sigma-Aldrich) and grown at 25°C in darkness for 5 d. A 5 mm- diameter agar plug from a 5 d old *F.*

graminearum or *B. cinerea* culture, or a 2 d old *R. solani* culture was inoculated on the opposite site of the CZ plate (Dubey et al., 2014; 2016). Mycelial growth rates of the pathogens were measured daily with a ruler. The mycoparasitic ability of *C. rosea* WT and mutants was evaluated by measurement of the *C. rosea* growth rate when overgrowing the pathogen. Overgrowth rates were measured 11 d after pathogen inoculation, meaning 4-7 d after hyphal contact of *C. rosea* and pathogen. The same experimental set-up was used for screening *in- vitro* antagonism of 53 *C. rosea* strains (Broberg et al., 2018). Here, CZ agar plates were prepared with the same composition as in Sigma-Aldrich and cultures were incubated at 20°C for 6 d before *F. graminearum* was inoculated at a 5 cm distance to the growing front of C. rosea. Both experiments were performed in five biological replicates.

Pathogen growth on *C. rosea chiC1* culture filtrates

Three 7 mm-diameter agar plugs from a 11 d old *C. rosea* culture was inoculated in 50 ml full strength PDB and grown in 25°C. After 3 d, the culture filtrate was harvested by double filter sterilization using a 0.45 µm cellulose acetate membrane. Twenty ml culture filtrate was inoculated with a 5 mm-diameter agar plug of a *F. graminearum, B. cinerea* or *R. solani* culture. Mycelial dry weight was recorded 9 d after pathogen inoculation, in triplicates.

Biocontrol assay on wheat plants

Wheat seeds of the cultivar 'Stava' were coated with *C. rosea* spores by application of a 10 ml solution with $5x10^7$ spores and rotary shaking at 120 rpm for 30 min. Per treatment and replicate, 15 spore-coated seeds were sawn into moistened sand and a 5 mm-diameter agar plug of a 4-d old *F. graminearum* culture was placed beside a group of three seeds (Dubey et al., 2014, 2016). Treatments included a healthy control (application of PDA plug without *F. graminearum*, not spore coated seeds), a disease control (not spore coated seeds), and biocontrol treatment coated with *C. rosea* WT or *C. rosea* knock-out mutant spores. Plants were grown in growth chambers for 17 d with a photoperiod of 12 h light (190 µmol m^2 s^{-1}) and 12 h darkness, 70 % relative humidity, in 15°C. Germination rate was assessed as 'germinated' or 'not germinated'. Disease severity was scored on a scale from 0-4, with 0 indicating a healthy plant and 4 indicating a dead plant. Scoring was performed by two independent persons and the experiments consisted of five biological replicates.

2.5. Gene expression analysis

Mycelium harvest

Dual cultures on solid medium were performed on PDA as described above. The mycelial interaction zone was harvested at hyphal contact by scratching mycelium from the cellophane membrane and immediately frozen in liquid nitrogen. The samples were processed by grinding of frozen mycelium in liquid nitrogen and RNA was extracted as described above.

RT-qPCR primers and conditions

qPCR primers were designed to be 20-22 bp in length with annealing temperatures of approx. 60°C (Table S1). For primer testing, PCR products from reactions using *C. rosea* genomic DNA as template were purified by adding 0.1 x volume of sodium acetate (pH 5.2; 3 M), 2.5 x volume of EtOH (95 %) and incubating at -20°C for 4 hours. DNA was collected by centrifugation and the pellet washed with 70 % EtOH. The pellet was dissolved in MilliQ H_2O and the concentration of purified DNA measured using a NanoDrop® spectrometer. Primer efficiencies (E) were calculated from Ct values obtained during RT-qPCR of a 10 x dilution series as $E = 10^{(-1/a)}-1$ with 'a' representing the slope of Ct values plotted over the decadic logarithm of the dilution factor. RT-qPCR was performed in an iQ5 system (BioRad) using following the instruction for Evagreen® dye provided by the company. Reactions were performed in 15 µl reaction volume, in five biological replicates and two technical replicates. Sample gene expression (test) in relation to a control (calibrator) was normalized by expression of the reference (ref) gene β-tubulin. Expression change was calculated as Ratio = $(E_{target})^{\Delta Ct \text{ target (calibrator – test)}}/(E_{ref})^{\Delta Ct \text{ ref (calibrator – test)}}$.

2.6. Statistical analysis

Datasets were analyzed using one way ANOVA in Minitab®. Pairwise comparisons indicated in the figures were performed using the Fisher's method at 95 % significance level. In addition, p- values were calculated by performing a two-tailed Student's *t*-test with unequal variance in MS Excel®.

3. Results

3.1. *Nlrl* expression is increased in dual culture interaction of *C. rosea* with *B. cinerea*

Modulation of gene expression allows mycoparasitic fungi to recognize, tolerate or antagonise their prey. To study whether expression patterns of *C. rosea chiC1*, *prz1*, *nlrl* or *mfs* (Table 1) were changed in response to fungal pathogens, *C. rosea* was inoculated in dual culture with *F. graminearum* or *B. cinerea* and the mycelial interaction front was harvested at hyphal contact. Gene expression was then studied using RT-qPCR. There was no significant change in gene expression of *chiC1*, *prz1* or *mfs* after hyphal contact with *F. graminearum* or *B. cinerea*, in comparison to *C. rosea* self-interaction (Figure 1A,C,D). Similarly, *nlrl* expression was not changed in response to *F. graminearum* but was 1.2- fold upregulated at hyphal contact with *B. cinerea* ($p = 0.03$; Figure 1B).

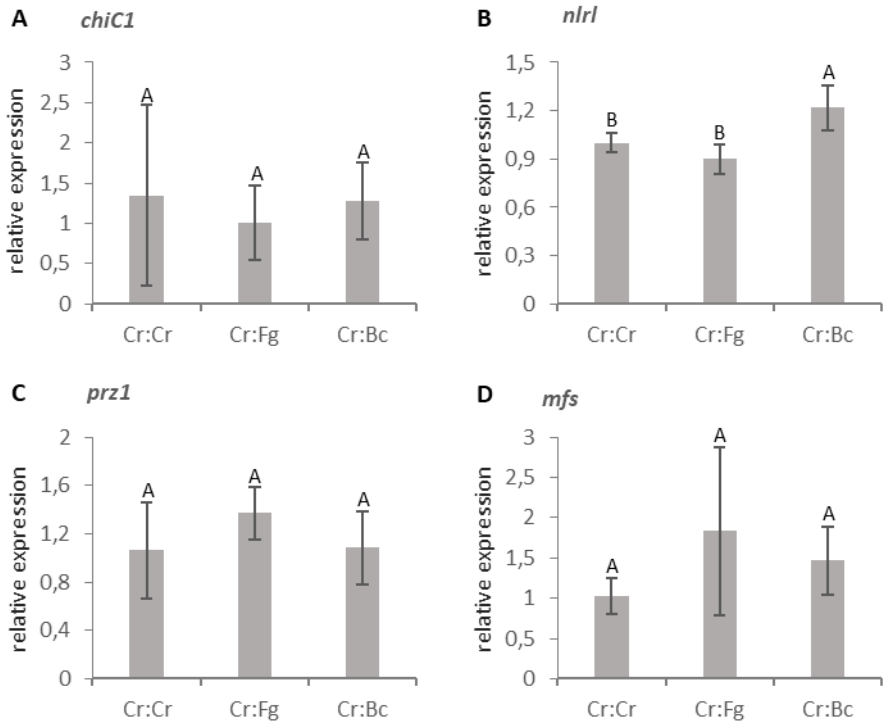


Figure 1. Relative gene expression of C. rosea during interaction with F. graminearum or B. cinerea. Gene expression change of *C. rosea* IK726 WT at hyphal contact with *F. graminearum* (Cr:Fg) or *B. cinerea* (Cr:Bc), in comparison to self-interaction (Cr:Cr). Expression profiles are normalized with expression of the β-tubulin gene. Error bars and statistical analysis using Fisher's method at 95 % confidence level are based on five biological replicates.

3.2. Creation and validation of *C. rosea* deletion strains ∆*chiC1,* ∆*prz1* and ∆*nlrl*

To further analyse the impact of the NLR-like receptor, the MFS transporter, the CHIC1 chitinase and the PRZ1 transcription factor on the interaction of *C. rosea* and *F. graminearum*, an *Ag. tumefaciens*-mediated transformation protocol was used to replace the respective gene with the *hygB* gene conferring resistance to Hygromycin. Expression vectors were created for all genes of *GOI*s using the MultiSite Gateway® technology, by inserting the *hygB* deletion cassette flanked by *GOI* upstream and downstream regions for homologous recombination (HR) into the agrovector pPM43GW (Fig. 1A). *Agrobacterium*-mediated transformation (ATMT) was performed and transformed colonies were selected on plates containing 200 µg/ml of hygromycin B. The number of observed colonies was 34, seven, five and 48 for *nlrl, mfs, chiC1* and *prz1*, respectively. To confirm that the deletion cassette was correctly integrated, PCR was performed with primers binding to a specific locus in the deletion cassette and approx. 100 bp outside of the

GOI flanking regions (Fig. 2A, Table S1). Fragments with the expected size were amplified in 20 out of 23 screened colonies from *nlrl* and *prz1* transformants and four out of five *chiC1* transformants (selection shown in Fig. 2B). No positive colony was detected for the *mfs* transformants (data not shown). ATMT of *C. rosea* was repeated with freshly transformed *Ag. tumefaciens* cells to generate *mfs* knock-out stains, but again, none out of 29 screened colonies showed correct PCR amplification from genomic DNA. Positive transformants of *nlrl, prz1* and *chiC1* were tested for mitotic stability and single spores were purified. Four individual transformants for each *GOI* were selected for gene expression analysis using RT-PCR with primers specific to the *GOI* and primers specific to β-*tubulin* to validate the cDNA quality (Figure 2A, Table S1). Gene expression was shown in *C. rosea* WT, as well was colony 4 of *chiC1* mutants, colony 8 of *nlrl* mutants and colony 21 of *prz1* mutants. A complete loss of *GOI* expression was seen for three individual transformants of all three candidate genes (Figure 2C). Thus, *C. rosea chiC1* transformants 1, 2, 5; *nlrl* transformants 6, 8, 15 and *prz1* transformants 6, 23, 24 were chosen for further phenotypical analysis.

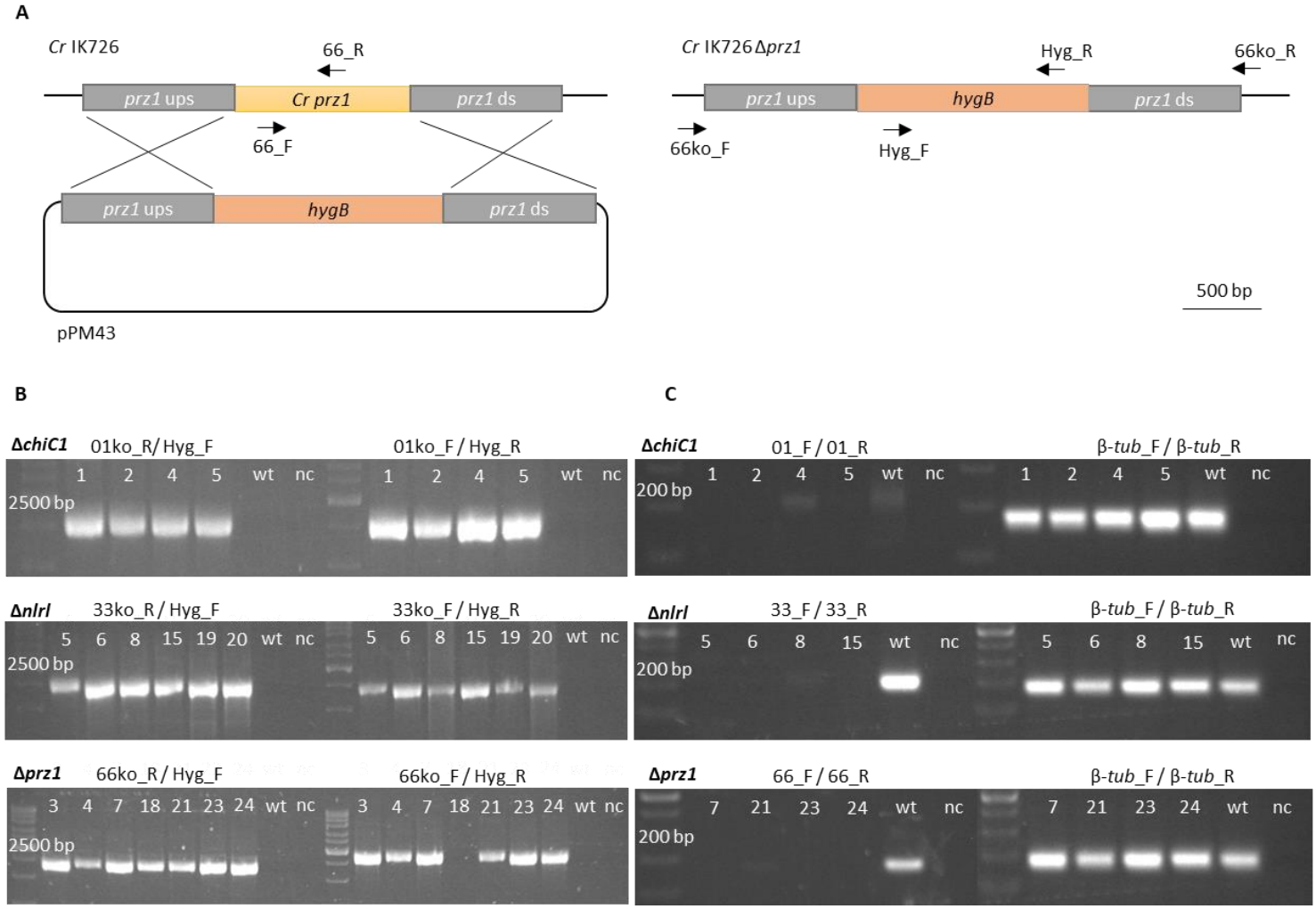


Figure 2. Validation of C. rosea gene deletion strains. (A) Illustration for creation of *C. rosea* knock-out mutants with *prz1* as an example. *GOI* was replaced with a hygromycin resistance cassette (*hygB*) originating from the pPm43GW plasmid via homologous recombination. The bar indicates the length for 500 bp and refers to the length of gene sequences, upstream (ups) and downstream (ds) regions. Arrows indicate the position of forward (F) and reverse (R) primers used for mutant validation. (B) PCR amplification of sequences specific to positive transformants using the indicated primers combinations. (C) Amplification with RT-PCR using the indicated primer combinations. wt, wild type; nc, negative control

3.2.1. *C. rosea* Δ*chiC1* growth rate is not carbon source-dependent, but *C. rosea* Δ*prz1* shows sensitivity to ionic and osmotic stresses

Gene deletion can lead to a general decrease in *C. rosea* growth rate but can also trigger increased sensitivity towards altered carbon sources, as well as ionic and osmotic stress inducers in the growth medium. To study the capability of *C. rosea* Δ*chiC1* to utilize different carbon sources as energy supply, *C. rosea* WT and three individual Δ*chiC1* transformants were inoculated on SMS medium with 1 % glucose, 0.5 % chitin or 0.9 % *F. graminearum* CWs as sole carbon source. Growth rates on SMS + glucose varied between 0.75- 0.88 mm/d (Figure 3A). Due to different growth rates between *C. rosea* transformants in standard SMS medium, growth in other conditions is given as growth change to purely show the effect of carbon source alteration. Generally, growth on chitin or *F. graminearum* CWs was around 6-fold faster than on SMS with glucose as carbon source. However, when compared to WT, chitin instead of glucose did not change Δ*chiC1* growth pattern, except for transformant 5 that showed significantly slower growth ($p = 0.01$; Figure 3B). Similarly, transformant 5 was the only knock-out strain that, compared to WT, showed decreased growth on medium with *F. graminearum* cell walls instead of glucose ($p = 0.003$; Figure 3C).

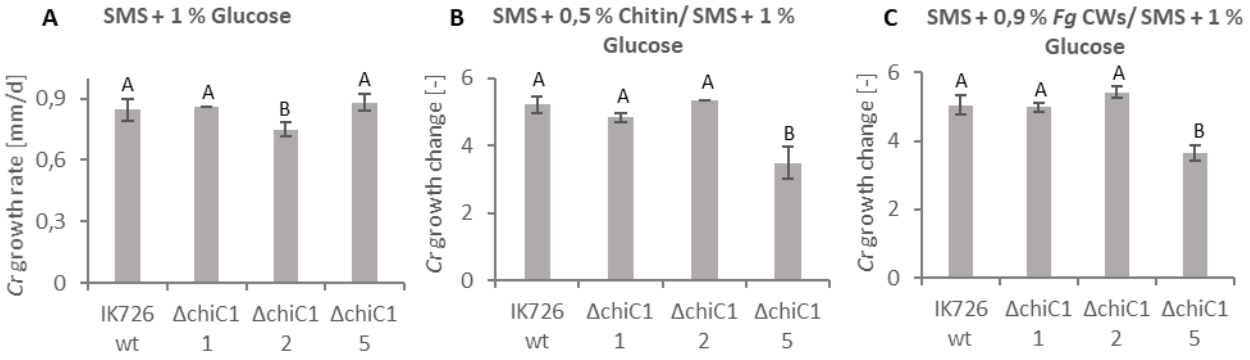


*Figure 3. C. rosea WT and Δ*chiC1* growth rates on different carbon sources.* (A) *C. rosea* growth rates on SMS medium with 1 % glucose. Due to differences in growth rates and to the detect the sole effect of carbon source change, *C. rosea* growth rate changes are given when glucose was replaced with (B) 0.5 % chitin or (C) 0.9 % *F. graminearum* CWs. N=3.

Due to the involvement of PRZ1 in intracellular calcium signalling, *C. rosea* Δ*prz1* growth rate was examined on Czapek Dox agar (Cz) with 400 mM $CaCl_2$, 10 mM $LiCl_2$ or 0.025 % of the osmotic stress inducer SDS. Growth rate of *C. rosea* WT and Δ*prz1* strains on standard Cz varied between 0.3-0.35 cm/d, while transformant 23 showed significantly reduced growth compared to the other strains ($p \leq 0.001$; Figure 4A). As motivated above, the effect of additional $CaCl_2$, $LiCl_2$ or SDS was then indicated as growth change compared to the growth rate on

standard Cz. Δ*prz1* transformants 7 and 24 showed reduced growth on additional SDS (p = 0.03), while Δ*prz1* 23 did not have significantly decreased growth compared to WT. Among them, Δ*prz1* 24 grew slower than Δ*prz1* 7 (Figure 4B). A similar growth change was observed on supplemented $LiCl_2$, although here only Δ*prz1* 24 differed significantly from the WT according to Fisher's test at 95 % significance, but not according to *t*-test analysis (p = 0.08; Figure 4D). Addition of $CaCl_2$ had a stronger growth inhibitory effect on *C. rosea* Δ*prz1* than on WT. Here, all Δ*prz1* strains showed consistently stronger reduced growth rates on $CaCl_2$ compared to WT and standard Cz with Fisher's test, but not with *t*-test analysis (p = 0.07; Figure 4C).

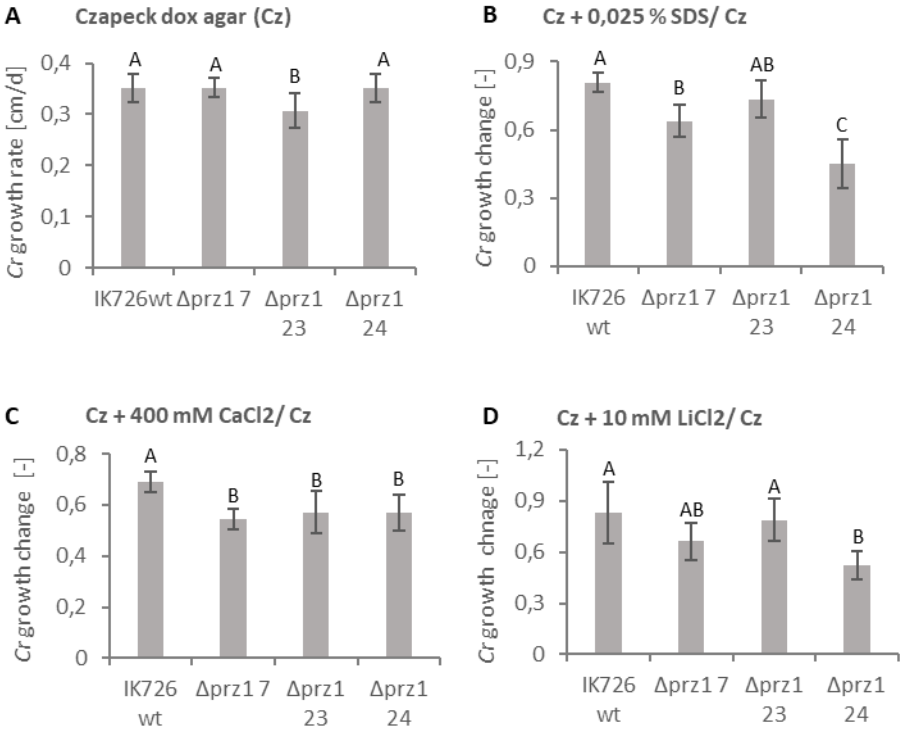


Figure 4. C. rosea WT and Δprz1 growth rates on ionic and osmotic stress inducers. (A) *C. rosea* growth rates on Czapek Dox agar (Cz). Due to differences in growth rates and to detect the sole effect of supplemented compounds, *C. rosea* growth rate changes are given under addition of (B) 0.025 % SDS or (C) 400 mM CaCl2 or (D) 10 mM LiCl2. (A) N=15; (B)-(D) N=3.

3.2.2. *F. graminearum* shows decreased mycelial growth in liquid culture with *C. rosea* Δ*chiC1* culture filtrates

Structural analysis of the *C. rosea* chitinase CHIC1 using the *SignalP-5.0*® and *SecretomeP 2.0* server indicated that translated CHIC1 is secreted from *C. rosea* cells through non- classical protein secretion. To investigate whether secreted CHIC1 can inhibit mycelial growth of different pathogens, culture filtrates of *C. rosea* WT and Δ*chiC1* were inoculated with agar plugs containing *F. graminearum*,

B. cinerea or *R. solani* mycelium. Pathogen growth was then assessed by mycelial harvest and measurement of the dry weight. Surprisingly, *F. graminearum* growth was reduced in Δ*chiC1* culture filtrates that do not contain CHIC1, compared to growth in WT culture filtrates where *chic1* is still expressed. There were further significant differences in growth reduction in culture filtrates of different Δ*chiC1* strains (p = 0.001; Figure 5A). No effect of C. *rosea* Δ*chiC1* culture filtrates on *B. cinerea* growth was observed (Figure 5B) and differences measured for *R. solani* growth are not trustworthy due to the absence of biological replicates for Δ*chiC1* 5 (Figure 5C).

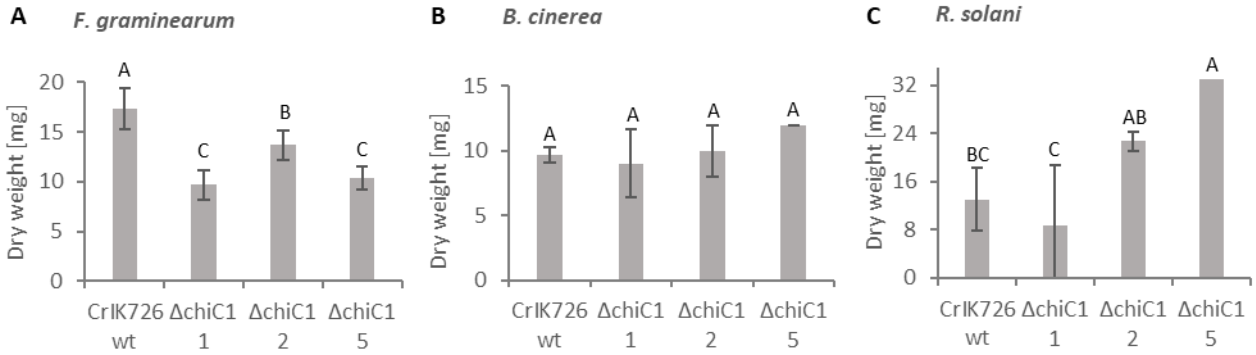


Figure 5. F. graminearum, B. cinerea and R. solani dry weight after cultivation in C. rosea Δ*chiC1 culture filtrates compared to WT.* Dry weight in mg of (A) *F. graminearum*, (B) *B. cinerea*, (C) *R. solani* after 9 d incubation in *C. rosea* culture filtrates. N=1-3

3.2.3. *F. graminearum* shows increased growth rate in dual culture interaction with *C. rosea* Δ*nlrl* compared to WT

Antagonism and mycoparasitism are important aspects of the biocontrol trait. One experimental approach to assess *in- vitro* antagonism of fungal BCAs are dual culture interaction assays. Inoculation of both BCA and pathogen on opposite sides of the same agar plate allows A) observation of pathogen growth rates when approaching the antagonist and B) measurements of BCA growth rates after hyphal contact of both species. Here, reduced pathogen growth rates indicate strong antagonistic behaviour of the BCA, while fast BCA growth rates after hyphal contact indicate strong mycoparasitism. To assess the antagonistic and mycoparasitic effect of *C. rosea* knock-out mutants compared to WT, dual culture interactions of IK726 WT, Δ*nlrl,* Δ*prz1* and Δ*chiC1* were performed towards *F. graminearum, B. cinerea* and *R. solani,* respectively. Presence of *C. rosea* WT did not reduce the growth rate of *F. graminearum* and *B. cinerea* significantly, when compared to growth rate of the pathogen in monoculture (*Fg* alone/ *Bc* alone, Figure 6, 7A). However, there was a significant reduction in *F. graminearum* growth rate in dual culture with *C. rosea* WT compared to *F. graminearum* growth in dual culture with another *F. graminearum* culture *(*p = 0.02; *Fg* + *Fg*; Figure 7A).

Secondly, presence of *C. rosea* WT reduced the growth rate of *R. solani* compared to *R. solani* growth in monoculture according to Fisher's analysis ($p \leq 0.05$), but not according to *t*-test ($p = 0.08$; Figure 7A). Comparing the antagonistic effect of *C. rosea* deletion mutants with WT did not reveal any consistent effects. Although faster *F. graminearum* growth was seen in dual cultures with transformants 5 and 15 of Δ*nlrl* strains, this was not observed for transformant 6 ($p = 0.03$; Figure 6, 7C). Strikingly, Δ*nlrl* 5 and Δ*nlrl* 15 did not affect the growth rate of *B. cinerea* or *R. solani* compared to WT (Figure 7C). No transformants of Δ*prz1* or Δ*chiC1* altered pathogen growth rates differently than *C. rosea* WT (Figure 7B,D).

Secondly, there was no significant effect of *C. rosea* overgrowth when comparing WT and deletion strains (data not shown).

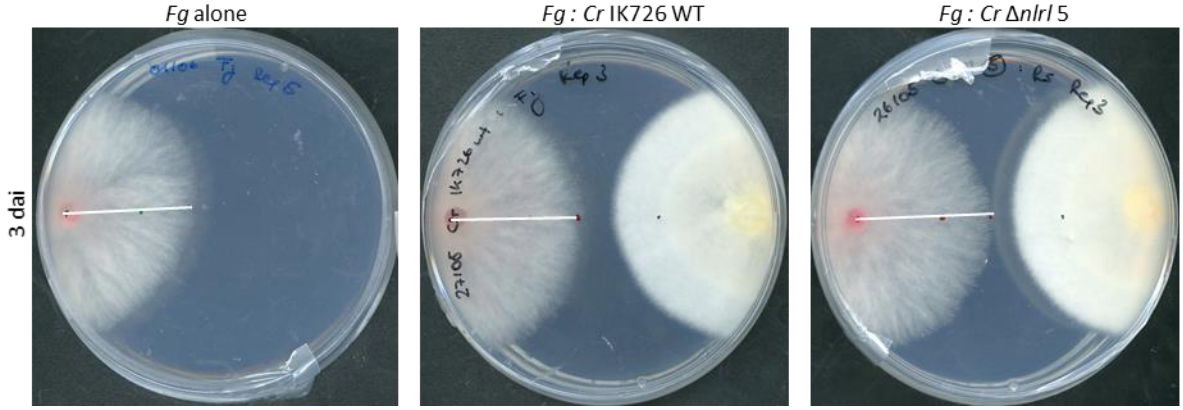


Figure 6. In-vitro antagonism of C. rosea Δ*nlrl in dual culture with F. graminearum. F. graminearum* mycelial growth in monoculture, dual culture with *Cr* IK726 WT or *Cr* Δ*nlrl 5* respectively 3 d after pathogen inoculation (dai). Pictures represent data shown in Figure 7C.

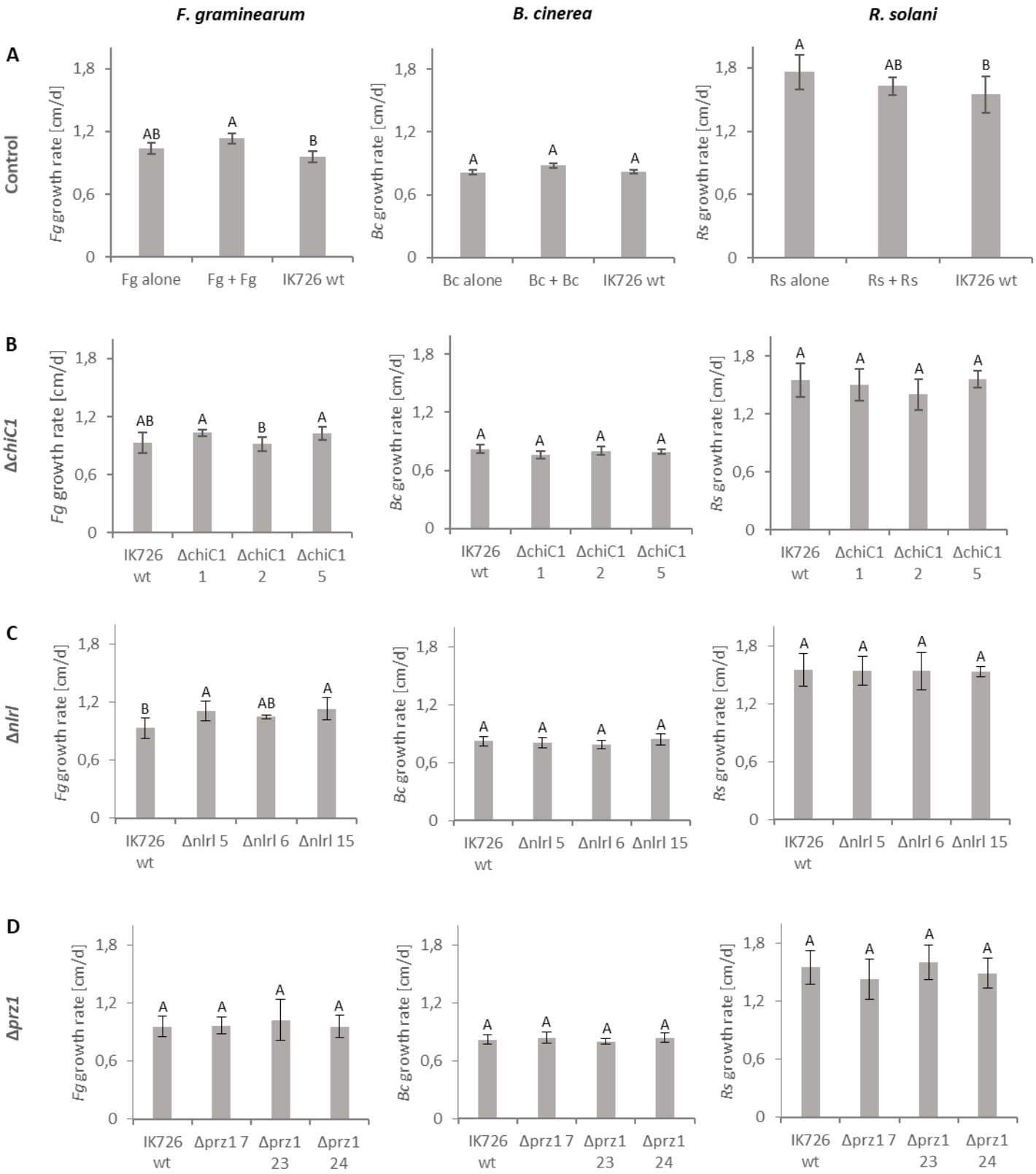


*Figure 7. Growth rates of F. graminearum, B. cinerea and R. solani in dual culture interaction with C. rosea WT, Δ*chiC1*, Δ*nlrl* and Δ*prz1*.* Growth rates of respective pathogens on Czapek dox agar. N=4-5 for each pathogen *C. rosea* combination. (A) Growth rates of respective pathogens in monoculture (e.g., *Fg* alone), dual culture with itself (e.g. *Fg* + *Fg*) and dual culture with *C. rosea* IK726 wt. Comparisons of pathogen growth rates in dual culture with *C. rosea* knock-outs are given in (B) Δ*chiC1*, (C) Δ*nlrl* and (D) Δ*prz1*.

3.2.4. Biocontrol effect of fusarium root rot in wheat was not altered upon treatment with *C. rosea* ∆*chiC1,* ∆*prz1* or ∆*nlrl* compared to IK726 WT

All candidate genes are putatively involved in the molecular response of *C. rosea* towards *F. graminearum* and may therefore play a role in biological control of FRR. Particularly *nlrl* is expectedly important here, as it was associated with the biocontrol trait *in- planta* during GWAS. To test the hypothesis of a contribution of the candidate genes to biocontrol of FRR, wheat seeds were coated with *C. rosea* WT, Δ*chiC1*, Δ*prz1* or Δ*nlrl* spores and sown in sand near an agar plug with actively growing *F. graminearum.* Development of FRR was assessed by counting of germinated seeds and scoring of disease symptoms on juvenile plants. Germination rate was strongly decreased in the disease control were no treatment with *C. rosea* spores was done. Notably, application of *C. rosea* WT spores increased the germination rate by more than 100 % ($p \leq 0.001$; Figure 8A). However, treatment with *C. rosea* Δ*chiC1, Δprz1 or* Δ*nlrl* spores did not significantly differ from the effect observed in WT (Figure 8 B-D), except for spores harvested from Δ*chiC1* 2 where a slightly increased germination rate was recorded that was not significant according to *t*-test (p= 0.1; Figure 8B).

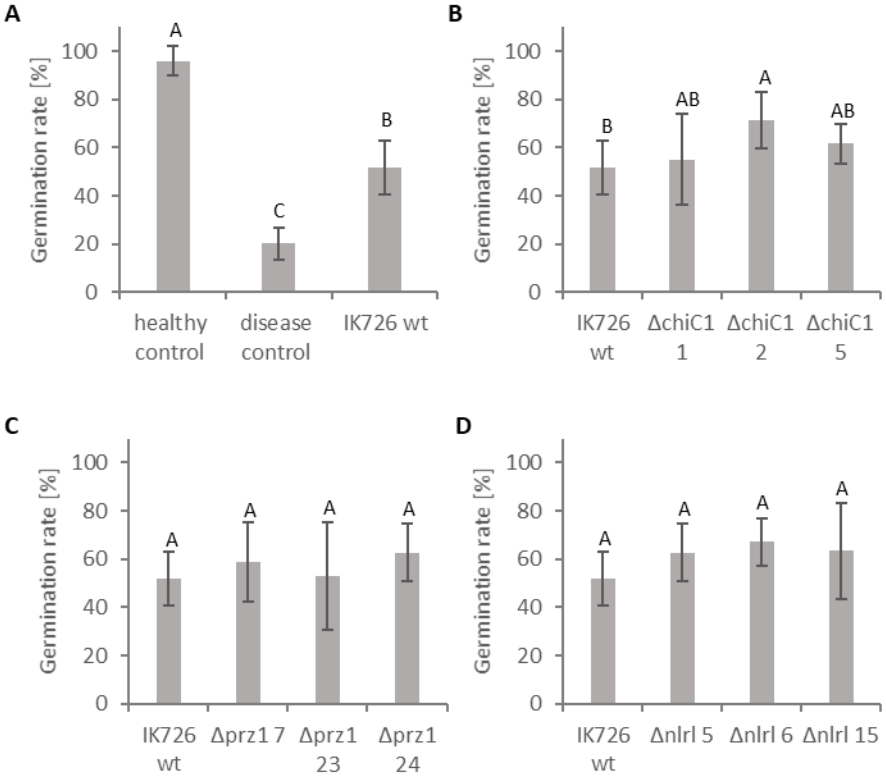


Figure 8. Germination rate of wheat seeds coated with C. rosea WT, ΔchiC1, Δprz1 or Δnlrl spores and exposed to F. graminearum. (A) Germination rate of the control treatments. Healthy control, not spore-coated, not exposed to *F. graminearum*; disease control, not spore-coated, exposed to *F. graminearum*; IK726 WT, coated with IK726 spores, exposed to *F. graminearum*. (B-D) Seeds coated with Δ*chiC1*, Δ*prz1* or Δ*nlrl*, respectively, and exposed *to F. graminearum*. For each treatment 15 seeds were scored per replicate. N = 5.

Similarly, a significant reduction in disease symptoms was observed in juvenile wheat plants treated with *C. rosea* WT spores ($p \leq 0.001$; Figure 9A). Deletion of neither *chiC1*, *prz1* nor *nlrl* altered *C. rosea* ability to supress FRR disease (Figure 9B-D).

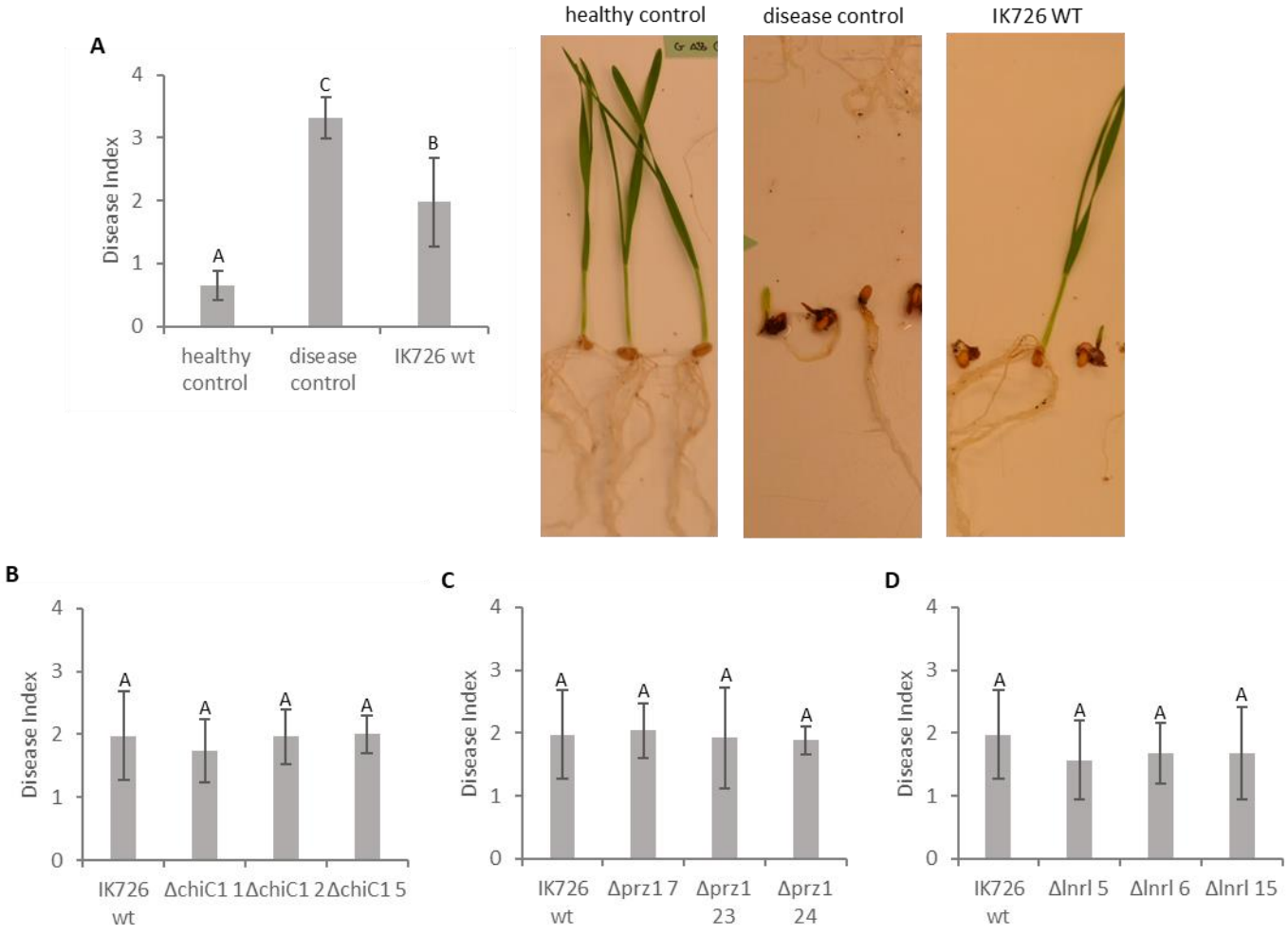


*Figure 9. Manifestation of FRR disease in juvenile wheat plats treated with C. rosea WT, Δ*chiC1*, Δ*prz1* or Δ*nlrl* spores.* Disease severity was assessed on a scale from 0-4, with 0 indicating healthy plants and 4 indicating germinated, but dead plants. (A) control treatments with representative pictures. (B) Δ*chiC1*. (C) Δ*prz1*. (C) Δ*nlrl*. For each treatment 15 seeds were scored per replicate. N=5.

3.3. Collection of *C. rosea* strains shows differences in *in-vitro* antagonism and mycoparasitism of *F. graminearum*

The biocontrol trait of *C. rosea* consists of different aspects, including antagonism and mycoparasitism of the prey. To gain solid understanding of *C. rosea* biocontrol, it is important to study each trait individually. One approach to determine genes involved in a specific trait are genome- wide association studies, where phenotypic data of a large number of individuals is compared with the genetic information of each individual. To identify genes involved in *C. rosea* antagonism of *F. graminearum,* 53 whole-genome sequenced *C. rosea* strains were screened on their ability to decelerate *F. graminearum* mycelial growth during *in-vitro* dual cultures and to overgrow *F. graminearum* cultures after hyphal contact. As illustrated in figure 10A, *F. graminearum* growth rates when approaching an actively growing *C. rosea* culture varied between 0.5-0.9 cm/d. According to pairwise comparison with Fisher's method at 95 % significance level, the presence of 31 *C. rosea* strains reduced *F. graminearum* growth rates significantly ($p \leq 0.005$) compared to *F. graminearum* growth rates in monoculture without *C. rosea* (Figure S2). With 44 % growth reduction *C. rosea* SHW-1-1 affected *F. graminearum* growth rates the most, while dual culture with *C. rosea* IK726 reduced *F. graminearum* growth rate by 20 %, compared to growth rate of *F. graminearum* in monoculture (*Fg* alone, Figure 10A, B). There was no significant difference between *F. graminearum* growth rates alone and when approaching another *F. graminearum* culture, eliminating that reduced growth rates are barely due to competition for space or nutrients (Figure 10A).

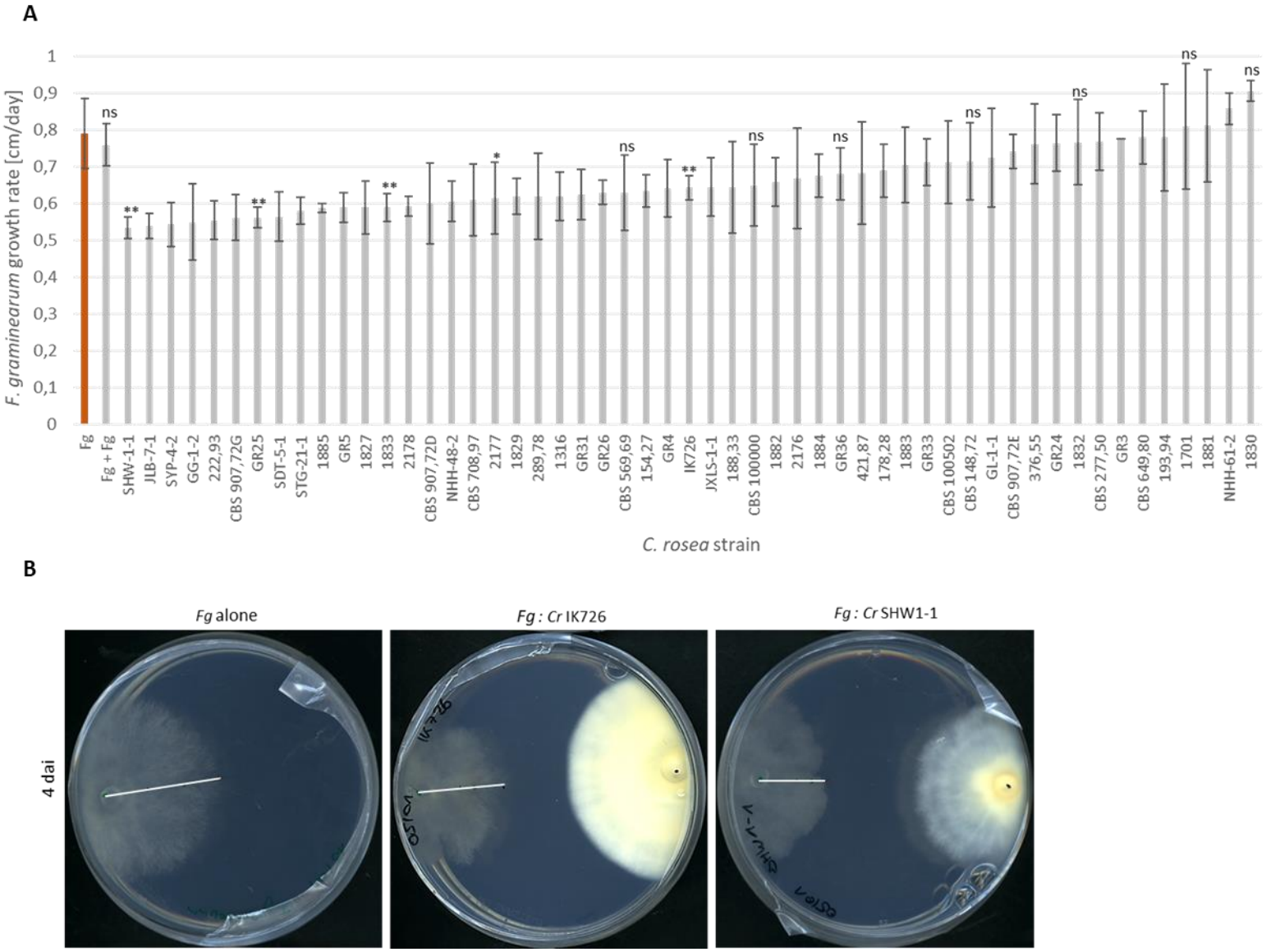


*Figure 10. In-vitro antagonism traits of 53 C. rosea strains in dual culture with F. graminearum. (*A) Growth rate of *F. graminearum* mycelium inoculated on the opposite side of an agar plate as the indicated *C. rosea* strain. Growth was measured 4 d after pathogen inoculation (dai). *Fg* represents the growth rate of *F. graminearum* without an antagonist on the plate. *Fg +Fg* represents the growth rate of *F. graminearum* when growing on the same plate with another *F. graminearum* culture. (B) Pictures of *F. graminearum* culture in monoculture, dual culture with *C. rosea* IK726 or *C. rosea* SHW1-1, respectively. Comparison with *Fg* control was calculated using two-tailed *t*-tests with unequal variance. Ns, non-significant (p>0.05); * (p>0.01); ** (p>0.001). Pairwise comparisons were performed using Fisher's method at 95 % significance level (Figure S2).

Secondly, *C. rosea* strains showed great variation in their ability to overgrow *F. graminearum,* ranging from growth rates of 0- 0.23 cm/d ($p \leq 0{,}005$; Figure 11). As *F. graminearum* overgrowth over itself is hard to assess, comparison were made in relation to the overgrowth rate of *C. rosea* IK726. Significant differences

between individual strains were observed (Figure S3). Notably, 9 *C. rosea* strains could not overgrow *F. graminearum* and there was no correlation of *C. rosea* growth and overgrowth rates (R^2= 0,03), indicating an additional genetic effect on overgrowth. Due to wide distribution in both datasets (Figure 10A, 11), the data can further be used for GWAS analysis of the *C. rosea in- vitro* antagonism trait.

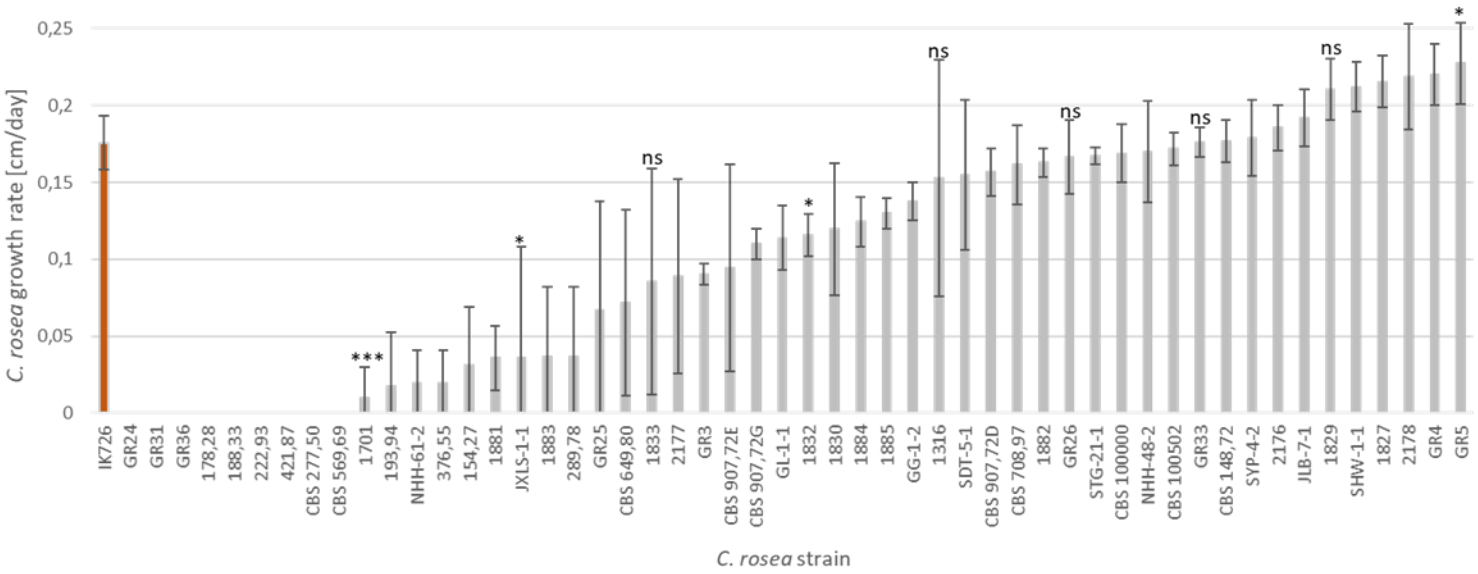


Figure 11. Overgrowth rate of 53 C. rosea strains in dual culture with F. graminearum. Growth rate of indicated *C. rosea* strain after mycelial contact with *F. graminearum*. Overgrowth was measured 11 d after pathogen inoculation. Comparison with *Cr* IK726 was calculated using two-tailed *t*-tests with unequal variance. Ns, non-significant (p>0.05); * (p>0.01); ** (p>0.001). Pairwise comparisons were performed using Fisher's method at 95 % significance level (Figure S3).

4. Discussion

Biological control of fungal plant pathogens by the mycoparasitic ascomycete *C. rosea* is a complex trait, mediated by a great number of genes (Sun et al., 2020). To improve applications of the *C. rosea* strain IK726, identification and functional characterization of many of those genes will be necessary. The increasing availability of genomic and transcriptomic information through sequencing techniques; as well as the combination with phenotypic data allows for indication of genes putatively involved in biocontrol (Karlsson et al., 2015; Nygren et al., 2018). Generating *C. rosea* deletion strains of identified genes then provides a powerful tool to confirm this function. However, gene deletion does not necessarily lead to an observable phenotype. This can be both due to functional redundancy within the gene family or due to limitations within the experimental setup (Fatema et al., 2018; Schöneberg et al., 2015).

In this study I generated the gene deletion mutants of three *C. rosea* genes- *chiC1,* encoding for a killer toxin-like chitinase; *prz1,* encoding for a transcription factor involved in stress-mediated calcium signalling and *nlrl,* putatively encoding for a receptor protein (Table 1; Figure 2). ATMT to additionally generate deletion strains of the *mfs* gene, encoding for an MFS transporter protein, did not result in correct integration of the Hyg resistance cassette and thus, the *mfs* gene could not be deleted. In filamentous fungi, efficiency of HR events is with 10-30 % low compared to other organisms. It has been shown that HR efficiency is negatively correlated with short recombination sites, as well as with an unfavourable position of the GOI in the target genome (Ding et al., 2019). It is therefore likely that low recombination efficiencies paired with suboptimal transformation conditions do not lead to an efficient number of positive transformants on selection plates.

The *C. rosea* genes *chiC1, prz1* or *nlrl* were expressed, but not induced, at hyphal contact with *F. graminearum,* but *nlrl* was induced at contact with *B. cinerea* (Figure 1). Expression of *chiC1* during *in- vitro* dual culture interaction has been studied previously by Nygren et al. (2018) where no expression change was reported towards *B. cinerea,* but a 50-fold increase in gene expression towards *F. graminearum.* During the experimental set-up from Nygren et al. (2018), the mycelial interaction zone was harvested 24 h after hyphal contact, instead of

directly at contact, as in this study. Hence, this data indicates that modulation of *C. rosea chiC1* expression occurs during contact with the prey, but not before.

In plants and animals, NLRs are important activators of downstream signalling cascades in response to non-self recognition (Uehling et al., 2017). Also in fungi, NLR homologs are receptor molecules that mediate cellular changes upon biotic interactions. An early upregulation of the *nlrl* gene in dual-culture interaction was therefore expected, however, we showed that *nlrl* expression was only induced at contact with *B. cinerea* but not with *F. graminearum.* Specificity in gene regulation towards different pathogens has been shown previously (Nygren et al., 2018) and is thought to be driven by e.g. different cell wall compositions of the prey or a different set of secondary metabolites involved in the particular interaction. Exclusive upregulation of *nlrl* expression at contact with *B. cinerea* could therefore indicate specific recognition of *B. cinerea* effector molecules by the NLRL protein examined in this study. Specificity in NLRL mediated recognition is also supported by the large structural diversity of the NLRL family in fungi (Dyrka et al., 2014), which allows for specialized protein- and nucleotide binding domains. *Nlrl* was identified during GWAS of the *C. rosea* biocontrol ability towards FRR (Dubey et al., unpublished), however no modulation in gene response was seen in dual culture interaction with the FRR causing agent *F. graminearum.* This, again, might show differently regulated gene responses during *in- vivo* biocontrol and *in- vitro* antagonism, but also points out some limitations of GWAS. Although GWAS successfully associates a given trait with the genomic composition of a species, it does not provide information whether differentially regulation of the identified genes is causing that trait (Tam et al., 2019).

Phenotypic analysis of *C. rosea* Δ*chiC1* showed differences in growth rate of transformant 2 on standard medium (Figure 3A). This may be caused by additional insertions of the gene disruption cassette and thus, from three individual transformants of one gene, the phenotype of the majority of transformants is to be trusted. It can therefore be concluded that there was no effect on *C. rosea* Δ*chiC1* growth rate when 0.5 % colloidal chitin or 0.9 % *F. graminearum* CWs were used as the sole carbon source (Figure 3B, C). In a gene expression analysis in *C. rosea* WT by Tzelepis et al. (2015), *chiC1* expression was increased upon 24 h growth in liquid SMS medium with 1 % colloidal chitin, suggesting a role in nutrient acquisition. Nevertheless, concurrent upregulation of other chitinase genes, such as *chiB2* or *chiA5,* shows functional redundancy within that gene family and could explain the absent phenotype in Δ*chiC1* compared to WT (Tzelepis et al., 2015). Surprisingly, *F. graminearum* growth was negatively affected in *C. rosea* Δ*chiC1* culture filtrates, while *B. cinerea* and *R. solani* growth rates did not differ significantly compared to WT (Figure 5). It would be expected that prey species of *C. rosea* show increased growth in the absence of killer-toxin like chitinases, as it has previously been reported for *B. cinerea* and *R. solani* growing in Δ*chiC2* culture filtrates (Tzelepis et al., 2015). However, deletion of *chiC1* might also trigger

increased production and secretion of other *C. rosea* chitinases to compensate for the loss-of function, which could explain inhibition of *F. graminearum* growth. Notably, the experimental evidence during gene expression analysis (Nygren et al., 2018), growth rate analysis by Tzelepis et al. (2015) and in this study (Figure 5), suggests involvement of CHIC1 during interaction with *F. graminearum* and CHIC2 during interaction with *B. cinerea* or *R. solani*. This confirms the proposed functional adaptation of killer toxin-like chitinases towards different prey species by Nygren et al. (2018).

PRZ1 is an important factor in the calcium-calcineurin signalling pathway and mediates Ca^{2+} homeostasis in the cytosol by inducing synthesis of transporter proteins that direct Ca^{2+} uptake from the cytosol into the cell organelles (Liu et al., 2015). One such Ca^{2+} transporter is encoded by the *pmc1* gene. *Pmc1* deletion strains of *S. cerevisiae* were shown to be specifically sensitive towards long-term Ca^{2+} stress and cell wall damaging (Matheos et al., 1997). In this study, Δ*prz1* strains showed reduced growth rates on 400 mM $CaCl_2$ or 0.025 % SDS (Figure 4), indicating a similar function of *prz1* in *C. rosea* Ca^{2+} signalling and stress response. The *prz1* homologs in *Aspergillus fumigatus* and *Magnaporthe oryzae* additionally play a role in chitin synthase expression and appressorium formation, respectively (Roy et al., 2020), suggesting diverse involvement of the PRZ1 transcription factor in *C. rosea* mycoparasitism.

The growth rate of *B. cinerea* and *R. solani* was not influenced during dual culture interaction on solid medium with *C. rosea* Δ*chiC1,* Δ*prz1* or Δ*nlrl* compared to WT, while *F. graminearum* growth rate was increased in dual culture with Δ*nlrl,* but not with Δ*chiC1* or Δ*prz1* (Figure 6). Similar to our results during gene expression analysis (Figure 1), NLRL appears to be involved in *C. rosea in-vitro* antagonism. However, *nlrl* expression was specifically induced upon interaction with *B. cinerea,* while dual culture with Δ*nlrl* exclusively increased *F. graminearum* growth rates. As previously reported by Fatema et al. (2018) gene expression in *C. rosea* can be culture medium- dependent, which might explain diverging results during gene expression studies on PDA medium and dual culture interactions on Cz medium. It is nevertheless surprising that dual culture with *C. rosea* IK726 WT did not influence *F. graminearum* or *B. cinerea* growth rates (Figure 6), particularly in comparison with our analysis of different *C. rosea* strains in dual culture with *F. graminearum,* where *F. graminearum* growth was significantly reduced in dual culture with IK726 WT (Figure 9).

Germination rate or disease establishment were not affected during biocontrol assays of FRR in wheat seeds or plants, when seeds were coated with Δ*chiC1,* Δ*prz1* or Δ*nlrl* spores instead of WT spores (Figure 7,8). GWAS analysis suggested an involvement of NLRL in biological control of FRR (Dubey et al., unpublished), hence, Δ*nlrl* mutants were expected to show reduced ability for biocontrol in our experimental set-up. Not much is known about NLRLs in *C. rosea*, however, due to large diversity and variability of that family shown in other fungal species, absent

phenotypes of *C. rosea* Δ*nlrl* in biocontrol assays may also be explained by functional redundancy within that protein family.

4.1. Outlook

Much of the here collected data points to a contribution of NLRL during *in- vitro* antagonism of *C. rosea*. To decipher this contribution, it may be useful to further characterize *C. rosea* NLRLs using phylogenetic analysis. Gene expression analysis of Δ*nlrl* could also be used to study NLRL signalling and identify genes involved in the response towards NLRL activation. To further analyse the unexpected decrease of *F. graminearum* growth rate in Δ*chiC1* culture filtrates and test the hypothesis that expression of other chitinase genes is upregulated in response to *chiC1* deletion strains, the chitinase activity of the culture filtrates could be measured. One of the main limitations in the here presented phenotyping assays is low sensitivity for knock-out mutants of functionally redundant genes. Generation of higher-order mutants would be possible and could be interesting for understanding the relation of *chiC1* and *chiC2* during antagonism and biocontrol.

5. References

Abrams, N., Badger, J., Robson, G. D., Wortman, J., & Nierman, W. (2005). Comparative analysis of programmed cell death pathways in filamentous fungi. *BMC Genomics, 6*, 177. doi:10.1186/1471-2164-6-177

Bowman, S., & Free, S. (2006). The structure and synthesis of the fungal cell wall. Bioessays 28, 799-808. *BioEssays, 28*, 799-808. doi:10.1002/bies.20441

Broberg, M., Dubey, M., Sun, M. H., Ihrmark, K., Schroers, H. J., Li, S. D., . . . Karlsson, M. (2018). Out in the cold: identification of genomic regions associated with cold tolerance in the biocontrol fungus *Clonostachys rosea* through genome-wide association mapping. *Front Microbiol, 9*, 2844. doi:10.3389/fmicb.2018.02844

Buist, G., Steen, A., Kok, J., & Kuipers, O. P. (2008). LysM, a widely distributed protein motif for binding to (peptido)glycans. *Mol Microbiol, 68*(4), 838-847. doi:10.1111/j.1365-2958.2008.06211.x

Demissie, Z., Witte, T., Robinson, K., Sproule, A., Foote, S., Johnston, A., . . . Loewen, M. (2020). Transcriptomic and exometabolomic profiling reveals antagonistic and defensive modes of *Clonostachys rosea* action against *Fusarium graminearum*. *Mol Plant-Microbe Interact, 33*. doi:10.1094/MPMI-11-19-0310-R

Ding, Y., Wang, K.-F., Wang, W.-J., Ma, Y.-R., Shi, T.-Q., Huang, H., & Ji, X.-J. (2019). Increasing the homologous recombination efficiency of eukaryotic microorganisms for enhanced genome engineering. *Applied Microbiology and Biotechnology, 103*(11), 4313-4324. doi:10.1007/s00253-019-09802-2

Dubey, M., Vélëz, H., Broberg, M., Jensen, D. F., & Karlsson, M. (2020). LysM proteins regulate fungal development and contribute to hyphal protection and biocontrol traits in *Clonostachys rosea*. *Front Microbiol, 11*, 679. doi:10.3389/fmicb.2020.00679

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*. *Mol Genet Genomics, 291*(2), 677-686. doi:10.1007/s00438-015-1139-y

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. Mol Plant Microbe Interact, 27*(7), 725-732. doi:10.1094/mpmi-12-13-0365-r

Dubey, M. K., Ubhayasekera, W., Sandgren, M., Jensen, D. F., & Karlsson, M. (2012). Disruption of the Eng18B ENGase gene in the fungal biocontrol agent *Trichoderma atroviride* affects growth, conidiation and antagonistic ability. *PloS one, 7*(5), e36152-e36152. doi:10.1371/journal.pone.0036152

Dyrka, W., Lamacchia, M., Durrens, P., Kobe, B., Daskalov, A., Paoletti, M., . . . Saupe, S. (2014). Diversity and variability of NOD-like receptors in fungi. *Genome Biology and Evolution, 6*, 3137-3158. doi:10.1093/gbe/evu251

Fatema, U., Broberg, A., Jensen, D. F., Karlsson, M., & Dubey, M. (2018). Functional analysis of polyketide synthase genes in the biocontrol fungus *Clonostachys rosea*. *Sci Rep, 8*(1), 15009. doi:10.1038/s41598-018-33391-1

Inglis, G., & Kawchuk, L. (2002). Comparative degradation of oomycete, ascomycete, and basidiomycete cell walls by mycoparasitic and biocontrol fungi. *Canadian journal of microbiology, 48*, 60-70. doi:10.1139/w01-130

Iqbal, M., Dubey, M., Broberg, A., Viketoft, M., Jensen, D. F., & Karlsson, M. (2019). Deletion of the nonribosomal peptide synthetase gene nps1 in the fungus *Clonostachys rosea* attenuates antagonism and biocontrol of plant pathogenic *Fusarium* and nematodes. *Phytopathology, 109*(10), 1698-1709. doi:10.1094/phyto-02-19-0042-r

Jensen, B., Knudsen, I. M. B., & Jensen, D. F. (2000). Biological seed treatment of cereals with fresh and long-term stored formulations of *Clonostachys rosea*: biocontrol efficacy against *Fusarium culmorum*. *European Journal of Plant Pathology, 106*(3), 233-242. doi:10.1023/A:1008794626600

Jia, C., Zhang, K., Zhang, D., Yu, Q., Xiao, C., Dong, Y., . . . Li, M. (2018). Effects of disruption of pmc1 in the tfp1Δ/Δ mutant on calcium homeostasis, oxidative and osmotic stress resistance in *Candida albicans*. *Mycopathologia, 183*(2), 315-327. doi:10.1007/s11046-017-0216-7

Karimi, M., De Meyer, B., & Hilson, P. (2005). Modular cloning in plant cells. *Trends Plant Sci, 10*(3), 103-105. doi:10.1016/j.tplants.2005.01.008

Karlsson, M., Durling, M. B., Choi, J., Kosawang, C., Lackner, G., Tzelepis, G. D., . . . Jensen, D. F. (2015). Insights on the evolution of mycoparasitism from the genome of *Clonostachys rosea*. *Genome Biol Evol, 7*(2), 465-480. doi:10.1093/gbe/evu292

Knudsen, I. M. B., Hockenhull, J., Jensen, D., Gerhardson, B., Hökeberg, M., Tahvonen, R., . . . Henriksen, B. (1997). Selection of biological control agents for controlling soil and seed-borne diseases in the field. *European Journal of Plant Pathology, 103*, 775-784. doi:10.1023/A:1008662313042

Kosawang, C., Karlsson, M., Vélëz, H., Rasmussen, P. H., Collinge, D. B., Jensen, B., & Jensen, D. F. (2014). Zearalenone detoxification by zearalenone hydrolase is important for the antagonistic ability of *Clonostachys rosea* against mycotoxigenic *Fusarium graminearum*. *Fungal Biology, 118*(4), 364-373. doi:https://doi.org/10.1016/j.funbio.2014.01.005

Kovalchuk, A., & Driessen, A. (2010). Phylogenetic analysis of fungal ABC transporters. *BMC Genomics, 11*, 177. doi:10.1186/1471-2164-11-177

Liu, S., Hou, Y., Liu, W., Lu, C., Wang, W., & Sun, S. (2015). Components of the calcium-calcineurin signaling pathway in fungal cells and their potential as antifungal targets. *Eukaryot Cell, 14*(4), 324-334. doi:10.1128/ec.00271-14

Loquet, A., & Saupe, S. (2017). Diversity of amyloid motifs in nlr signaling in fungi. *Biomolecules, 7*. doi:10.3390/biom7020038

Matheos, D. P., Kingsbury, T. J., Ahsan, U. S., & Cunningham, K. W. (1997). Tcn1p/Crz1p, a calcineurin-dependent transcription factor that

differentially regulates gene expression in *Saccharomyces cerevisiae*. *Genes Dev, 11*(24), 3445-3458. doi:10.1101/gad.11.24.3445

Nielsen, L. K., Jensen, J. D., Nielsen, G. C., Jensen, J. E., Spliid, N. H., Thomsen, I. K., . . . Jørgensen, L. N. (2011). Fusarium head blight of cereals in Denmark: species complex and related mycotoxins. *Phytopathology, 101*(8), 960-969. doi:10.1094/phyto-07-10-0188

Nygren, C. M. R., Eberhardt, U., Karlsson, M., Parrent, J. L., Lindahl, B. D., & Taylor, A. F. S. (2008). Growth on nitrate and occurrence of nitrate reductase-encoding genes in a phylogenetically diverse range of ectomycorrhizal fungi. New Phytologist, 180(4), 875-889. doi:https://doi.org/10.1111/j.1469-8137.2008.02618.x

Nygren, K., Dubey, M., Zapparata, A., Iqbal, M., Tzelepis, G. D., Durling, M. B., . . . Karlsson, M. (2018). The mycoparasitic fungus *Clonostachys rosea* responds with both common and specific gene expression during interspecific interactions with fungal prey. *Evol Appl, 11*(6), 931-949. doi:10.1111/eva.12609

Ravnskov, S., Jensen, B., Knudsen, I. M. B., Bødker, L., Funck Jensen, D., Karliński, 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 and Biochemistry, 38*(12), 3453-3462. doi:https://doi.org/10.1016/j.soilbio.2006.06.003

Roohparvar, R., De Waard, M. A., Kema, G. H. J., & Zwiers, L.-H. (2007). MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. *Fungal Genetics and Biology, 44*(5), 378-388. doi:https://doi.org/10.1016/j.fgb.2006.09.007

Roy, A., Kumar, A., Baruah, D., & Tamuli, R. (2020). Calcium signaling is involved in diverse cellular processes in fungi. *Mycology, 12*. doi:10.1080/21501203.2020.1785962

Schöneberg, A., Musa, T., Voegele, R., & Vogelgsang, S. (2015). The potential of antagonistic fungi for control of *Fusarium graminearum* and *F. crookwellense* varies depending on the experimental approach. *Journal of Applied Microbiology, 118*. doi:10.1111/jam.12775

Stenberg, J., Sundh, I., Becher, P., Björkman, C., Dubey, M., Egan, P., . . . Viketoft, M. (2021). Correction to: When is it biological control? A framework of definitions, mechanisms, and classifications. *Journal of Pest Science, 94*. doi:10.1007/s10340-021-01386-z

Sun, Z.-B., Li, S.-D., Ren, Q., Xu, J.-L., Lu, X., & Sun, M.-H. (2020). Biology and applications of *Clonostachys rosea*. *Journal of Applied Microbiology, 129*(3), 486-495. doi:https://doi.org/10.1111/jam.14625

Tam, V., Patel, N., Turcotte, M., Bossé, Y., Paré, G., & Meyre, D. (2019). Benefits and limitations of genome-wide association studies. *Nature Reviews Genetics, 20*(8), 467-484. doi:10.1038/s41576-019-0127-1

Tzelepis, G., Dubey, M., Jensen, D. F., & Karlsson, M. (2015). Identifying glycoside hydrolase family 18 genes in the mycoparasitic fungal species *Clonostachys rosea*. *Microbiology (Reading), 161*(7), 1407-1419. doi:10.1099/mic.0.000096

Uehling, J., Deveau, A., & Paoletti, M. (2017). Do fungi have an innate immune response? An NLR-based comparison to plant and animal immune systems. *PLOS Pathogens, 13*, e1006578. doi:10.1371/journal.ppat.1006578

Xu, R., & Li, Q. (2008). Protocol: Streamline cloning of genes into binary vectors in *Agrobacterium* via the Gateway(R) TOPO vector system. *Plant methods, 4*, 4. doi:10.1186/1746-4811-4-4

Xue, A. G. (2003). Biological control of pathogens causing root rot complex in field pea using *Clonostachys rosea* strain ACM941. *Phytopathology, 93*(3), 329-335. doi:10.1094/phyto.2003.93.3.329

6. Supplementary material

6.1. Supplementary tables

Table S 1. PCR primers for Gateway® cloning, mutant validation, and RT-qPCR

Candidate genes	Primer name	Primer sequence (5'->3')	Tm [°C]	Amplicon length [nt]
CRV2T00017633	7633ds_F	GGGGACAACTTTGTATAATAAAGTTGTACGAGCATGTTGTAGCAGTTTGGA	67,8	842
	7633ds_R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCGAGGTGATGGAGGAGGGAGAG	74,8	
	7633up_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCCAGAGCGCCCACGAATC	72	908
	7633up_R	GGGGACAACTTTGTATAGAAAAGTTGGGTGTGGAAAAGGCACAAAGCGGACA	71	
	7633ko_R	TGCTGTAGGGCTATCAAATGGG	55,7	2400
	7633ko_F	GAGAGCACTGGCCAAGGTCAC	56	2500
	7633_F	TCCTGGCCCGTAGAGAAGAGAGTA	57,4	191
	7633_R	CGCATGTGGCAGGTATTCCC	57,5	
CRV2T00015724	5724ds_F	GGGGACAACTTTGTATAATAAAGTTGTACCGGGCCGAGCTTGAGACAG	70,5	643
	5724ds_R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGATCTGGTCCCCGTTGTGCT	74	
	5724ups_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGCAATCCCCAGCCAAACTCAC	71,6	929
	5724ups_R	GGGGACAACTTTGTATAGAAAAGTTGGGTGTCCAGAAGATCCACCGCCAGTC	72,5	
	5724ko_R	CGCTAAAAACAGGAGGAGACGA	55,2	2400
	5724ko_F	GGATAAAACTAACGGGGAGGGA	55,2	2200
	5724_F	TCCAAGATGAAAACCCCAAAGAGG	59,3	171
	3524_R	CGATGCCGAATTCTGTCTCAAGC	59,7	

CRV2T00011101	1101ds_F	GGGGACAACTTTGTATAATAAAGTTGTAAGGCATCATGGCGGACAAAGAC	68,8	922
	1101ds_R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTGATCCGGGCTGGAATACAACC	73,4	
	1101ups_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCTCTGGGGGATTGCTTGG	72,9	972
	1101ups_R	GGGGACAACTTTGTATAGAAAAGTTGGGTGCGGAGGTGATTTCTCGGATGAT	70,2	
	1101ko_R	TGCCATCACCGCCATCTATGC	60,2	2500
	1101ko_F	GTCGCCCGAGCTCATCAACC	59,1	2500
	1101_F	GGACCCGAGCTGCCATCATC		157
	1101_R	TCATCGTCAAACGACACCCACTG		
CRV2T00002266	2266ds_F	GGGGACAACTTTGTATAATAAAGTTGTACGCTGTGGCACTCGGGTAGAT	69,8	1070
	2266ds_R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTCGTGCGTGTTGATAAGGGC	72,6	
	2266ups_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCGGCGTCTGGGTCCTTTTTA	72	761
	2266ups_R	GGGGACAACTTTGTATAGAAAAGTTGGGTGCCGATGGCCGCTTGATGGTG	72,1	
	2266ko_R	TGCCTCGAGGGTAAGCGGTAGA	59,7	2300
	2266ko_F	ACGCCAGACCGACGCCAC	59,1	2600
	2266_F	CAAGGGGTGCGAGATGAAAGC		156
	2266_R	CATGTCCCGTTCCAGCACTTGAG		
	Hyg_F	GCGCGCAATTAACCCTCAC	59	With ko_R
	Hyg_R	GAATTGCGCGTACAGAACTCC	61	With ko_F
	β-tub_F	TTCAGACCGGTCAGTGCGTA	60	190
	β-tub _R	GCCAGAAAGCAGCACAAT	55	

Above listed primers were used for this project. Underlined sequences represent Gateway® specific sequences. Amplicon length of knockout_R primers are concerning PCR amplifications in combination with Hyg_F primer and vice-versa.

6.2. Supplementary figures

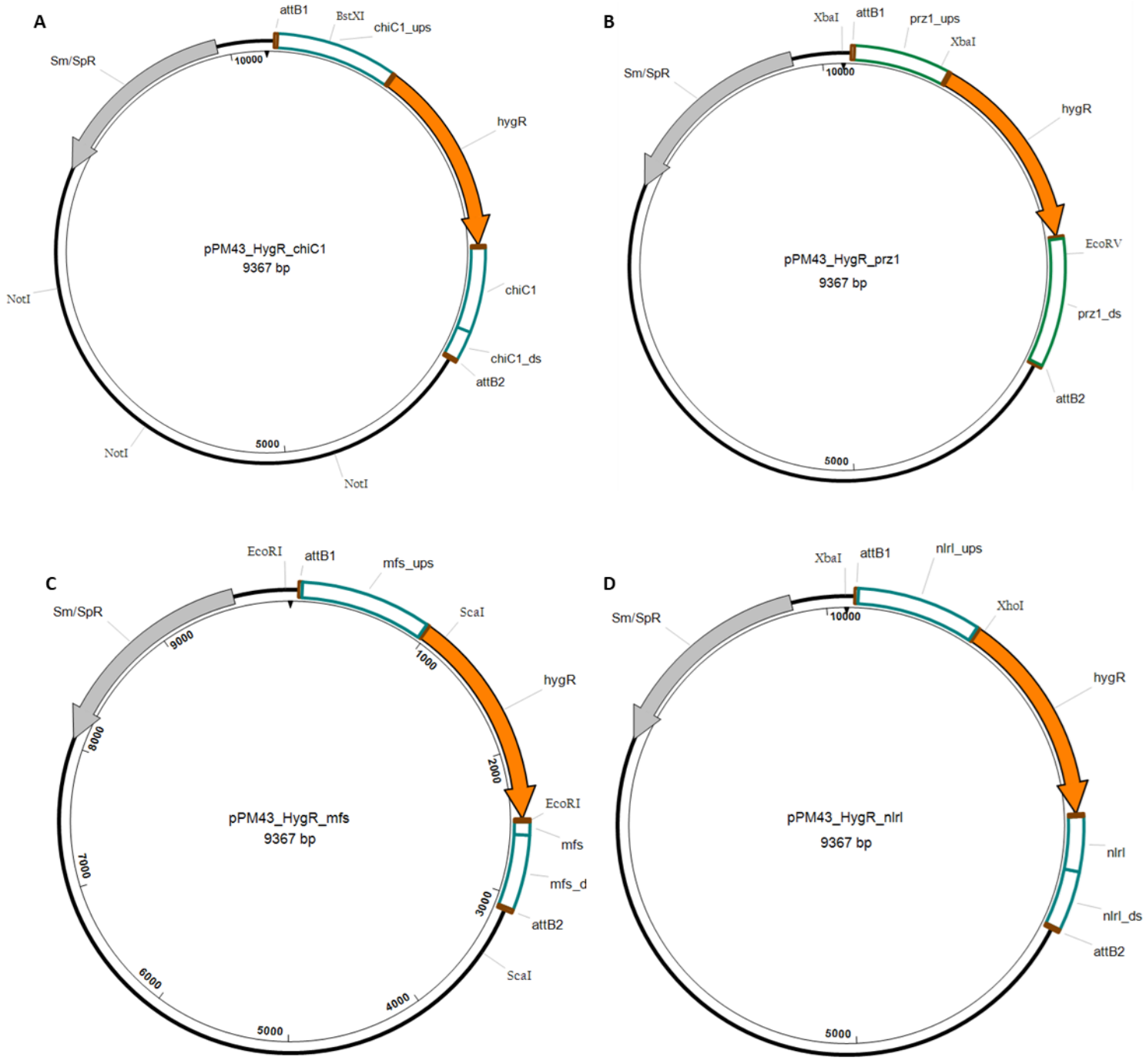


Figure S 1 Gateway® expression vectors for ATMT of C. rosea. Expression vectors carrying a spectinomycin resistance cassette (SpR) and a hygromycin resistance cassette (hygR), flanked by *C. rosea* upstream and downstream regions of the *GOI*. Enzymatic restriction sites used for plasmid validation are indicated, as well was Gateway® specific regions of homologous recombination. Plasmid were created for deletion of the *C. rosea* genes (A) *chiC1*, (B) *prz1*, (C) *mfs* and (D) *NLRL*.

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Cr strain	N	Mean	Grouping
1830	4	0.9063	A
NHH-61-2	3	0.8583	A B
1881	4	0.8125	A B C D
1701	5	0.8100	A B C
Fg	5	0.7900	A B C D E
193.94	5	0.7800	A B C D E F
CBS 649,80	5	0.7800	A B C D E F
GR3	3	0.7750	A B C D E F G
CBS 277,50	4	0.7687	B C D E F G
1832	3	0.7667	A B C D E F G H I J K L
GR24	5	0.7650	B C D E F G
376.55	4	0.7625	B C D E F G J
Fg + Fg	5	0.7600	B C D E F G J
CBS 907,72E	3	0.7417	B C D E F G H I J K L M
GL-1-1	5	0.7250	B C D E F G H I J K L M
CBS 148,72	5	0.7150	C D E F G H I J K L M N
GR33	2	0.7125	B C D E F G H I J K L M N O P Q R S T U
CBS 100502	4	0.7125	B C D E F G H I J K L M N O
1883	5	0.7050	C D E F G H I J K L M N O
178.28	5	0.6900	C D E F G H I J K L M N O R
421.87	3	0.6833	C D E F G H I J K L M N O P Q R S T U V
GR36	4	0.6813	D E F G H I J K L M N O P Q R S T U
1884	4	0.6750	E F G H I J K L M N O P Q R S T U V
1882	3	0.6583	E F G H I J K L M N O P Q R S T U V W
CBS 100000	5	0.6500	G H I J K L M N O P Q R S T U V W
188.33	5	0.6450	G H I J K L M N O P Q R S T U V W
JXLS-1-1	5	0.6450	G H I J K L M N O P Q R S T U V W
IK726	4	0.6438	G H I J K L M N O P Q R S T U V W
GR4	3	0.6417	F G H I J K L M N O P Q R S T U V W
154.27	5	0.6350	J K L M N O P Q R S T U V W
GR26	5	0.6300	I L M N O P Q R S T U V W
CBS 569,69	5	0.6300	H I K L M N O P Q R S T U V W
GR31	5	0.6250	M N O P Q R S T U V W
1316	5	0.6200	M N O P Q R S T U V W
289.78	5	0.6200	M N O P Q R S T U V W
1829	5	0.6200	M N O P Q R S T U V W
2177	5	0.6150	M N O P Q R S T U V W
CBS 708,97	5	0.6100	M N O P Q R S T U V W
NHH-48-2	4	0.6062	M N O P Q R S T U V W
CBS 907,72D	5	0.6000	N O P Q R S T U V W
2178	4	0.5938	N O P Q R S T U V W
2176	3	0.5917	M N O P Q R S T U V W
GR5	5	0.5900	O P Q R S T U V W
1833	5	0.5900	O P Q R S T U V W
1827	5	0.5900	O P Q R S T U V W
1885	2	0.5875	M N O P Q R S T U V W
STG-21-1	4	0.5812	O P Q R S T U V W
SDT-5-1	5	0.5650	U V W
GR25	4	0.5625	R S T U V W
CBS 907,72G	2	0.5625	N O P Q R S T U V W
222.93	5	0.5550	Q T U V W
GG-1-2	4	0.5500	P Q S T U V W
SYP-4-2	4	0.5437	V W
JLB-7-1	5	0.5400	W
SHW-1-1	5	0.5350	W

Means that do not share a letter are significantly different.

Figure S 2. Pairwise comparison with Fisher's method at 95 % confidence for *F. graminearum* growth rate dataset represented in Figure 10.

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Strains	N	Mean	Grouping
GR5	5	0.2274	A
GR4	2	0.2200	A B C D E F G
2178	4	0.2188	A B
1827	5	0.21533	A B
SHW-1-1	5	0.21200	A B D F
1829	5	0.2105	A B D F
JLB-7-1	5	0.19200	A B C D E F G H
2176	4	0.18542	A B C D E F G H
SYP-4-2	4	0.1787	A B C D E F G H
CBS 148,72	5	0.17679	B C D E F G H
GR33	2	0.17619	A B C D E F G H I J
IK726	3	0.1756	A B C D E F G H I
CBS 100502	4	0.17179	B C D E F G H I
NHH-48-2	4	0.1700	B C D E F G H I
CBS 100000	5	0.16886	B C D E F G H I
STG-21-1	4	0.16708	B C D E F G H I
GR26	5	0.1664	F G H I
1882	3	0.16270	B C D E F G H I J
CBS 708,97	4	0.1614	D E F G H I J
CBS 907,72D	5	0.15667	C E G H I J
SDT-5-1	5	0.1550	H I J
1316	5	0.1527	H I J
GG-1-2	2	0.1375	H I J K
1885	2	0.1300	H I J K L M
1884	4	0.12437	I J K
1830	4	0.1196	I J K L M
1832	3	0.11587	I J K L M
GL-1-1	5	0.1138	J K L M
CBS 907,72G	2	0.1100	I J K L M
CBS 907,72E	3	0.0944	K L M
GR3	3	0.09028	K L M N O P Q
2177	3	0.0889	K L M N O P Q
1833	5	0.0853	K L M N
CBS 649,80	5	0.0719	M N O P Q R
GR25	4	0.0667	L M N O P Q R S
289.78	5	0.0367	Q R S T
1883	5	0.0367	P Q R S T
JXLS-1-1	5	0.0360	O P Q R S T
1881	4	0.0357	N O P Q R S T
154.27	5	0.0310	R S T
NHH-61-2	4	0.0196	S T
376.55	4	0.0196	S T
193.94	5	0.0175	S T
1701	5	0.0100	T
GR36	4	0.000000	T
GR31	5	0.000000	T
GR24	5	0.000000	T
CBS 569,69	5	0.000000	T
CBS 277,50	4	0.000000	T
421.87	3	0.000000	T
222.93	3	0.000000	T
188.33	5	0.000000	T
178.28	5	0.000000	T

Means that do not share a letter are significantly different.

Figure S 3. Pairwise comparison with Fisher's method at 95 % confidence for *F. graminearum* overgrowth dataset represented in Figure 11.

7. Acknowledgements

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