Transcriptomic and Exometabolomic Profiling Reveals Antagonistic and Defensive Modes of *Clonostachys rosea* Action Against *Fusarium graminearum*

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The mycoparasite *Clonostachys rosea* ACM941 is under development as a biocontrol organism against *Fusarium graminearum*, the causative agent of Fusarium head blight in cereals. To identify molecular factors associated with this interaction, the transcriptomic and exometabolomic profiles of *C. rosea* and *F. graminearum* GZ3639 were compared during coculture. Prior to physical contact, the antagonistic activity of *C. rosea* correlated with a response heavily dominated by upregulation of polyketide synthase gene clusters, consistent with the detected accumulation of corresponding secondary metabolite products. Similarly, prior to contact, trichothecene gene clusters were upregulated in *F. graminearum*, while those responsible for fusarielin and fusarin biosynthesis were downregulated, correlating with an accumulation of trichothecene products in the interaction zone over time. A concomitant increase in 15-acetyl deoxynivalenol-3-glucoside in the interaction zone was also detected, with *C. rosea* established as the source of this detoxified mycotoxin. After hyphal contact, *C. rosea* was found to predominantly transcribe genes encoding cell wall-degradation enzymes, major facilitator superfamily sugar transporters, anion:cation symporters, as well as alternative carbon source utilization pathways, together indicative of a transition to necrotrophism at this stage. *F. graminearum* notably activated the transcription of phosphate starvation pathway signature genes at this time. Overall, a number of signature molecular mechanisms likely contributing to antagonistic activity by *C. rosea* against *F. graminearum*, as well as its mycotoxin tolerance, are identified in this report, yielding several new testable hypotheses toward understanding the basis of *C. rosea* as a biocontrol agent for continued agronomic development and application.

Keywords: antagonism, biocontrol agent, *Clonostachys rosea*, deoxynivalenol, detoxification, *Fusarium graminearum*, glycosylation, polyketides

The genus *Clonostachys rosea* (Schroers et al. 1999) includes a number of endophytic soil-borne ascomycete strains recognized for their ability to promote plant growth or to protect crop yields from pathogens and mycotoxin contamination, or both. Some of the plant pathogens reportedly controlled by *C. rosea* include *Fusarium* spp., *Alternaria* spp., *Botrytis* spp., and *Pythium tracheiphilum* (Lysøe et al. 2017; Sutton et al. 1997; Zhang et al. 2008). Strain *C. rosea* ACM941, isolated from pea plants in Manitoba, Canada in 1994 (Hue et al. 2009), is a biocontrol agent patented for use against *Fusarium graminearum* (Xue 2002). *F. graminearum* is the leading cause of Fusarium head blight (FHB) in North America, and *C. rosea* has been shown to reduce the FHB index by 46% and deoxynivalenol (DON) levels by 33% compared with controls (Xue et al. 2014). However, the molecular basis of its necrotrophic mycoparasitism against *F. graminearum* and its mycotoxin tolerance have remained largely enigmatic.

Experimental evidence and comparative genome analyses indicate that secondary metabolites play a central role in *C. rosea* mycoparasitism (Karlsson et al. 2015, 2017). For example, 15 secondary metabolites were isolated from *C. rosea* YRS-06, of which tetrahydrotrichodimer ether, dihydrotrichodimer ether A, dihydrotrichodimer ether B, TMC-151C, and TMC-151E displayed promising antibacterial activity (Zhai et al. 2016). *Sclerotinia sclerotiorum* growth inhibitory molecules, mainly composed of nonribosomal peptides, were also characterized from *C. rosea* BAFC3874 (Rodríguez et al. 2011). A recent study also showed that deletion of a nonribosomal peptide synthase gene (*NRPS1*) significantly weakens *C. rosea* IK726 nematocidal property and its in-plant biocontrol activity against foot rot disease caused by *F. graminearum* (Iqbal et al. 2019). With respect to FHB, *C. rosea* ACM941 secretes *F. graminearum* growth inhibitory molecules and predicted associated biosynthetic gene clusters were described (Demissie et al. 2018).

Additionally, *C. rosea* likely employs cell wall-degrading enzymes (CWDEs) to facilitate necrotrophism. Mycoparasitism of *Botrytis allii* by *C. rosea* was attributed to both low-molecular weight secreted metabolites and CWDEs, in particular β -(1,3)-glucanases and chitinases (Pachenari and Dix 1980). Scanning

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and transmission electron microscopy images of *C. rosea* interacting with *B. cinerea* (Yu and Sutton 1997; Li et al. 2002) also showed penetration of the *B. cinerea* hyphae by *C. rosea*, presumably enabled by enzymatic degradation, leading to cytoplasmic organelle disintegration. The CWDE associated with the endochitinase gene *Chi67-1* (isolated from *C. rosea* 67-1 [Sun et al. 2015]) has been functionally characterized and directly linked to *C. rosea* biocontrol properties. Contrary to this, deletion of a pectate lyase gene *PEL12* in strain IK726 failed to attenuate its mycoparasitic activity against *B. cinerea* (Atanasova et al. 2018). These mixed results highlight that the antagonistic mode of *C. rosea* is complex, likely involving both secondary metabolites and CWDEs.

Mycotoxin tolerance is an important trait of mycoparasitizing organisms, enabling their survival and neutralizing toxigenic fungal defense metabolites. Transcriptomic profiling (Kamou et al. 2016; Kosawang et al. 2014), gene-deletion experiments (Dubey et al. 2014), and comparative genomic analysis (Karlsson et al. 2015, 2017) of *C. rosea* have suggested that mycotoxin tolerance in *C. rosea* is likely a result of ATP-binding cassette (ABC) transporter-mediated efflux. In this regard, *C. rosea* grown in medium amended with fumonisin B1 showed no sign of growth retardation or any evidence of degraded or detoxified fumonisin B1, leading to the conclusion that its tolerance involves cellular clearance, likely by ABC-transporter proteins (Chatterjee et al. 2016). In addition, expression of the ABC-transporter gene *pdv5* increased upon *C. rosea* IK726 exposure to zearalenone (ZEA) (Kosawang et al. 2014) and *pdv5* deletion reduced ZEA tolerance (Dubey et al. 2014). Similarly, deletion of *pdv5* or mutations of key residues in its active site weakened yeast tolerance toward DON-type mycotoxins (Gunter et al. 2016). However, evidence suggests that enzymatic activity may also play a role in mycotoxin detoxification by *C. rosea*. For example, despite the evidence suggesting that *C. rosea* IK726 tolerance toward ZEA is mediated by PDR5 (Dubey et al. 2014; Kosawang et al. 2014), its detoxification was previously shown to be catalyzed by the ZEA hydrolase enzyme ZHD101 (Takahashi-Ando et al. 2002). Similarly, although a 33% reduction in DON levels has been achieved by *C. rosea* ACM941 in field and greenhouse experiments (Xue et al. 2014), it is not known if this is a result of *F. graminearum* growth inhibition or DON detoxification or both.

Toward addressing these gaps in our understanding of the mechanisms underlying the biocontrol activity of *C. rosea* ACM941 against *F. graminearum* GZ3639 as well as its trichothecene tolerance, an experimental coculturing system using plate confrontation assays was set up to compare both secondary metabolite excretion and gene transcription between interacting species and monoculture controls. Presented here are the results of transcriptomic and metabolomics profiling analyses that were used to compare the interspecies responses during confrontation, both before and after contact (BC and AC, respectively). The upregulation of secondary metabolite gene cluster expression and resulting metabolite accumulation in the interaction zone were found to occur BC, followed by a transition to necrotrophic growth and associated enzymatic expression of *C. rosea* AC. Together these findings highlight signature molecular mechanisms underlying the antagonistic effects and mycotoxin tolerance of *C. rosea*, raising an array of novel hypotheses for future testing and application in agricultural agronomy.

RESULTS

Phenotypic evaluation of the *C. rosea* and *F. graminearum* colony confrontation.

Fungal confrontation experiments showed that mycelium growth of the *F. graminearum* edge directly opposing the

C. rosea growing edge slowed significantly after 5 days of coculture. Instead, the *F. graminearum* leading edge was found to be growing vertically and laterally. In contrast, *C. rosea* grew steadily before reaching the *F. graminearum* edge and continued to grow over it (Supplementary Fig. S1).

C. rosea and *F. graminearum* RNA-Seq analysis and validation.

Two reference genomes of *C. rosea*—strains IK726 (Karlsson et al. 2015) and CBS125111 (available in the Joint Genome Institute [JGI] MycoCosm database)—and a de novo—assembled transcript database of strain *C. rosea* ACM941 are available (Demissie et al. 2018). In this report, the IK726 genome-derived transcripts were used as a reference for transcript abundance quantification for two purposes, i.e., to be consistent with a recently reported similar study (Nygren et al. 2018) and to compare the response of the two strains. Of the 14,628 *C. rosea* transcripts, 1,145 transcripts were differentially regulated in *C. rosea* (with ≥ 2.0 -fold expression, P value < 0.05), with 561 downregulated and 584 upregulated, respectively, before the two mycelia made contact (BC) (Fig. 1A). The number of differentially regulated *C. rosea* transcripts after the *C. rosea* and *F. graminearum* mycelia made contact (AC) was 2,082 (with ≥ 2.0 -fold expression, P value < 0.05), of which 940 were down-regulated and 1,142 were up-regulated (Fig. 1B). On the other hand, RNA-Seq analysis of *F. graminearum* cocultured with *C. rosea* revealed 864 and 882 genes were down- and up-regulated BC more than two-fold, respectively, compared with the control (P value < 0.05), while 1,009 and 765 genes were down- and up-regulated AC, respectively (Supplementary Table S1).

To confirm the veracity of the RNA-Seq expression data, quantitative PCR (qPCR) primers were designed against candidate *C. rosea* transcripts whose expression was either not changed or was up-regulated or downregulated in one or both stages (Supplementary Table S2). With the exception of *BN869_T00007232_1*, whose transcript abundance remained unchanged in the AC RNA sample using qPCR, the RNA-Seq expression profiles of all transcripts correlated well with those arising from qPCR (Fig. 2). Therefore, the RNA-Seq analysis results were deemed sufficiently reliable to represent the biological events occurring during the *C. rosea* and *F. graminearum* interaction and were advanced for further detailed analysis. Similarly, selected *TRI* (trichothecene) genes were used to validate RNA-Seq results of *F. graminearum* using droplet digital PCR (ddPCR) and were also found to corroborate the expression data (Fig. 3; Supplementary Fig. S2).

On the assumption that *C. rosea* mycoparasitism against *F. graminearum* is likely determined by upregulated transcripts, the remaining analysis was largely focused on these groups. A total of 361 (Supplementary Table S3) and 638 (Supplementary Table S4) transcripts showed significant upregulation BC and AC (with ≥ 2.5 -fold expression at P value < 0.05), respectively, of which only 52 were common (Supplementary Table S5). This lack of transcript commonality suggests divergence in the *C. rosea* responses at the BC and AC stages. This was further supported by the distribution of gene ontology (GO) terms associated with transcripts upregulated at the BC and AC stages. In particular, 24 (6%) transcripts with GO terms related to small metabolite biosynthesis were up-regulated at the BC stage, while only five ($< 1\%$) transcripts with similar GO terms were up-regulated ≥ 2.5 -fold in *C. rosea* AC (Supplementary Fig. S3). This implies that secondary metabolism biosynthesis is initiated early in the interaction, likely involving both “first line” offensive and defensive mechanisms from *C. rosea*. Further investigation of the expression of the top 100 upregulated transcripts from each timepoint (BC and AC), of which only 10 were in common, further emphasized this (Fig. 4A;

Supplementary Tables S2 and S3). Notably, CWDEs were substantially upregulated AC, while polyketide synthase (PKS) homolog transcripts were detected BC (details presented below). Together, these findings underscore a substantial change in the type of transcripts being upregulated at the two stages and suggest that the *C. rosea* mode of antagonism shifts, depending

on its proximity to *F. graminearum*. A similar effect was observed for *F. graminearum*, with only five of the top 100 upregulated genes, putatively encoding glutathione *S*-transferase, cytochrome b-5 reductase, and hypothetical proteins, upregulated in both stages of the interaction (Fig. 4B; Supplementary Table S1).

Fig. 1. Differential regulation of *Clonostachys rosea* ACM941 transcripts during confrontation with *Fusarium graminearum*. Volcano plots of differential gene expression arising from *C. rosea* versus *F. graminearum* coculture relative to *C. rosea* monoculture **A**, before the two colonies touched each other and **B**, after the two colonies touched each other. Volcano plots were obtained using DESeq2 software.

Fig. 2. Validation of RNA-Seq results by quantitative PCR (qPCR). **A**, Results arising for selected unchanged, upregulated, and downregulated transcripts before and **B**, after contact. RNA-Seq outcomes are represented by black bars, qPCR outcomes are represented by open bars. Cr07230 = BN869_T00007230_1, Cr07231 = BN869_T00007231_1, Cr07232 = BN869_T00007232_1, Cr07233 = BN869_T00007233_1, Cr07234 = BN869_T00007234_1, Cr07235 = BN869_T00007235_1, Cr07236 = BN869_T00007236_1, Cr07653 = BN869_T00007653_1, Cr08207 = BN869_T00008207_1, Cr05310 = BN869_T00005310_1, Cr04860 = BN869_T00004860_1, Cr10434 = BN869_T00010434_1, and Cr08191 = BN869_T00008191_1.

Extracellular metabolite accumulation validates *C. rosea* and *F. graminearum* in BC signature secondary metabolite gene cluster expression.

Three gene clusters with putative roles in secondary metabolite biosynthesis were up-regulated in *C. rosea* cocultured with *F. graminearum* at the BC stage (Supplementary Table S3).

Fig. 3. RNA-Seq expression results for *Fusarium graminearum* trichothecene gene cluster before contact with *Clonostachys rosea*, with validation using droplet digital PCR (ddPCR). Fungal *βtub* and *EF1α* genes were used as internal controls for normalization of transcripts. Three biological replicates of each treatment were assayed. Only *TRI8*, *TRI4*, and *TRI1* were assayed by ddPCR.

Two of these clusters were anchored by PKS homolog transcripts, including *BN869_T00012010_1*, which was expressed approximately 16-fold higher (Fig. 5), *BN869_T00013405_1*, expressed 4.2-fold higher, and *BN869_T00010693_1* and *BN869_T00010694_1*, expressed 2.2- and 2.6-fold higher, respectively (Table 1). qPCR analysis of these genes validated their RNA-Seq expression (Fig. 5; data not shown). Notably, transcripts *BN869_T00010693_1* and *BN869_T00010694_1* putatively encode homologs of a highly reducing PKS (77% similarity) and a nonreducing PKS (78% similarity), respectively. These were identified, by BLAST analysis against the UniProt database and FungiSMASH search, as SorB and SorA homologs of the bisorbicillinoids gene cluster, respectively (Guzmán-Chávez et al. 2017). Other members of the gene cluster were similarly upregulated (Table 1). The gene cluster anchored by the polyketide homolog *BN869_T00012010_1* (Fig. 5) also contained epoxide hydrolase-, asparagine synthase-, and cytochrome p450-encoding transcripts, along with two transcripts with unknown function that were also upregulated. A third potential secondary metabolism-related gene cluster upregulated at the BC stage was anchored by the major facilitator superfamily (MFS) transporter protein homolog *BN869_T00007234_1* that was up-regulated 125-fold (Fig. 2). However, although neighboring genes were also upregulated, fulfilling gene cluster category criteria, a putative function for this cluster cannot be assigned at this time.

While, the *C. rosea* genome is not sufficiently well-annotated to enable informed gene function predictions, in comparison, the *F. graminearum* genome is very well-annotated, enabling prediction and correlation of differentially regulated gene clusters with metabolites. Sieber et al. (2014) had identified 67 predicted secondary metabolism gene clusters (C01 to C67) by combining functional enrichment analysis of the *F. graminearum* genome with supporting coexpression data. RNA-Seq expression data of *F. graminearum* challenged by *C. rosea* showed that 15 of

Fig. 4. Comparative expression profile of top 100 upregulated transcripts. **A**, *Clonostachys rosea* and **B**, *Fusarium graminearum*, before (log2FC-BC) and after (log2FC-AC) contact. Only 10 transcripts were upregulated in both stages. Transcripts and their expression profile are provided in Supplementary Table S1.

the 67 gene clusters were significantly differentially regulated between treatments (Supplementary Table S6). Gene clusters responsible for biosynthesizing the fusarielins (C60 [Sørensen et al. 2012]), fusarins (C42 [Niehaus et al. 2013]), malinochrome (C63 [Oide et al. 2015]), and an unknown metabolite possibly related to iron transport (C22) were all downregulated at the BC stage. In addition, at least a subset of genes within the uncharacterized clusters C06 (containing *NRPS16*, *NRPS19*) and C07 were also downregulated BC. In contrast, the trichothecene (C23) (Fig. 3) (as well as the unlinked *TRI1* and *TRI101* genes), ZEA (C15), aurofusarin (C13 [Frandsen et al. 2011]) gene clusters and the two culmorin biosynthetic genes were up-regulated in the BC samples, while the gramillin gene cluster (C02 [Bahadoor et al. 2018]) was up-regulated both BC and AC. Two gene clusters, C28 (carotenoid [Jin et al. 2010]) and the uncharacterized C25, were downregulated AC with *C. rosea*.

To validate the differential expression of the above gene clusters with their predicted secreted metabolites, the exometabolites of *C. rosea* and *F. graminearum* isolated from cocultured plates compared with that of their respective monoculture

conditions were profiled. Data preprocessing yielded a total of 140 variables (representing pseudomolecular ions) that were detected above thresholding limits. The data were modeled by principal component analysis to check for sample outliers, showing clear separation between the four different sample classes in the first two principal components and explaining the majority of the variance in the model (Supplementary Fig. S4). A correlation analysis was performed to group associated pseudomolecular ion variables (protonated mass, adducts, neutral losses, fragments) based on expression levels and retention times, reducing the complexity of the data set down to 70 variable groupings. Pairwise comparisons were carried out on representative variables between monoculture and associated coculture interaction zone data sets, yielding a specific list of variables with significant differences in fold change (*P* value < 0.05) associated with differential metabolite accumulation in the interaction zone at all timepoints during the time course. The greatest amount of observed variance in secreted metabolite accumulation occurred between sample classes in those extracted from agar plugs harvested after the two colonies made

Fig. 5. A, Expression of a *Clonostachys rosea* polyketide-containing gene cluster by RNA-Seq, with validation by quantitative PCR (qPCR) before and B, after contact. *C. rosea* β Act and *EF1 α* genes were used as internal controls for normalization of transcripts. RNA-Seq outcomes are represented by black bars, qPCR validation is represented by open bars. Cr12005 = BN869_T00012005_1, Cr12006 = BN869_T00012006_1, Cr12007 = BN869_T00012007_1, Cr12008 = BN869_T00012008_1, Cr12009 = BN869_T00012009_1, Cr12010 = BN869_T00012010_1, Cr12011 = BN869_T00012011_1, and Cr12012 = BN869_T00012012_1.

Table 1. Expression of a putative *Clonostachys rosea* trichodimerol/bisorbicillinoid gene cluster detected by RNA-Seq^a

Transcript ID	Annotation	Fold change (<i>P</i> value)	
		Before contact	After contact
BN869_T00010687_1	sor8	2.037 (9.52E-15)	1.784 (2.92E-52)
BN869_T00010688_1	sor3	1.567 (2.08E-07)	1.793 (9.57E-49)
BN869_T00010689_1	sor6	2.404 (2.51E-33)	1.711 (1.33E-134)
BN869_T00010690_1	Esterase	1.931 (1.64E-14)	1.854 (2.78E-60)
BN869_T00010691_1	sor4	0.923 (0.406422)	0.804 (5.58E-08)
BN869_T00010692_1	sor5	2.045 (7.97E-20)	1.036 (0.253954)
BN869_T00010693_1	sor2	2.165 (5.00E-18)	0.869 (0.013191)
BN869_T00010694_1	sor1	2.564 (3.44E-32)	0.811 (9.03E-09)

^a Fold change values in bold type are significant at *P* value < 0.05, while italicized fold change values are not (*P* value > 0.05).

contact. All AC samples were subjected to a cluster analysis and an associated heat map of log-scaled metabolite accumulation was generated to visualize and compare trends in metabolite accumulation occurring between the monocultures and within the coculture interaction zones (Fig. 6). Exometabolites in the monoculture strains formed two clearly distinct clades, emphasizing the lack of overlapping metabolite expression and secretion by *C. rosea* and *F. graminearum*. At the same time, metabolite production from the interaction zones consisted of a mixture of metabolites observed from the two monoculture strains, along with variables that were unique to the interaction zone and absent from monoculture strains.

Annotations were possible for a number of *C. rosea* secondary metabolites based on published data (Zhao et al. 2016). Trichodimerol and associated bisorbicillinoid ethers (such as dihydro- and tetrahydrotrichodimer ethers) as well as the polyketides TMC-151A, TMC-151C, TMC-151E, and other associated analogs were observed in *C. rosea* monoculture-secreted metabolite profiles and *C. rosea*-*F. graminearum* interaction zones. Based on pseudomolecule ion peak area, relative accumulation levels were found to steadily increase over the experimental time course as cultures matured, diffusing through the agar from *C. rosea* to *F. graminearum* in the confrontation zone (Fig. 7A to D). Fluctuations in secondary

Fig. 6. Metabolic profile of *Clonostachys rosea* and *Fusarium graminearum* strains after 7-day coculture. Heat map comparison of autoscaled discriminatory variables representing secondary metabolites that accumulated by the after-contact stage (day 7) between *C. rosea* ACM941 (Cr) and *F. graminearum* Fg3639 (Fg) monocultures and their respective interaction zones in coculture (Cr-I and Fg-I). Representative metabolite variables expressed as retention time_{mass/charge ratio} (RT_{m/z}) or by a designation (15ADON-3G = 15-ADON-3-Glc, TDO = trichodimerol, TDO-associated = trichodimerol associated, TRI-associated = trichothecene associated). The black arrow highlights 15-ADON and the purple arrow 15-ADON-3-Glc.

metabolite production were also observed in *F. graminearum* monocultures compared with *C. rosea*–*F. graminearum* cocultures. Accumulation of fusarin, 15-acetyl-DON (15-ADON) and other associated trichothecenes were observed in *F. graminearum* monocultures at the AC stage (i.e., by day 7), confirming their production from *F. graminearum*. Significant fold changes in 15-ADON accumulation were observed in the interaction zone of the cocultures compared with *F. graminearum* monocultures at sampling timepoints both BC and AC, as predicted by BC differential *TRI* gene expression observed in the transcriptomic analyses (Fig. 3; Supplementary Fig. S1). Differential production of ZEA, culmorin, and aurofusarin, as suggested by the RNA-Seq data (Supplementary Table S6), was confirmed by extracted ion chromatograms of the ultra-high performance liquid chromatography-high resolution mass spectrometer (UPLC-HRMS) profiles to occur AC in the coculture interaction zones adjacent to *F. graminearum* compared with *F. graminearum* monocultures (and was absent in *C. rosea* monocultures). Gramillin-associated pseudomolecular ion signals were not detected by UPLC-HRMS profiling, despite upregulation of its gene cluster (Supplementary Table S6), which is likely an artifact of the extraction process, as gramillins are not soluble in ethyl acetate.

C. rosea detoxifies 15-ADON by glycosylation.

The mycotoxin tolerance observed in ACM941 and other *C. rosea* strains has been attributed to active efflux via ABC transporter proteins (Dubey et al. 2014; Karlsson et al. 2015). Therefore, the detection of 15-ADON-3-*O*-glucoside (15-ADON-3-Glc) in the *C. rosea* and *F. graminearum* confrontation zone of coculture plates was unexpected (Figs. 6 and 8).

Indeed, 15-ADON-3-Glc was absent in both of the monoculture controls over the duration of the time course. Confirmation of the identification of 15-ADON-3-Glc was carried out by tandem mass spectrometry (MS/MS) experiments, in which pseudomolecular fragment ions matched those of published data (Schmeitzl et al. 2015). Similar to 15-ADON, 15-ADON-3-Glc increased steadily in abundance over the time course in the confrontation zone, initiated BC, with concentrations consistently greatest at the *C. rosea* colony periphery, compared with that of *F. graminearum*. Taken together, these accumulation patterns suggest that while 15-ADON was secreted by *F. graminearum* and diffused toward the *C. rosea* periphery, it is transformed to 15-ADON-3-Glc by *C. rosea*, which subsequently diffuses back across the confrontation zone. In a separate experiment, when *C. rosea* was challenged with differing concentrations of pure 15-ADON, production of 15-ADON-3-Glc through glycosylation of 15-ADON by *C. rosea* was confirmed (Supplementary Fig. S5), albeit glycosylation occurred to a lesser extent than observed in the confrontation plates. This data raises the novel hypothesis that the 15-ADON glycosylation reaction is mediated by an endogenous glycosyltransferase from *C. rosea*, whose upregulation may involve dynamic signals arising from the *F. graminearum* secretome.

C. rosea triggers expression of cell-wall degradation and enhanced competitive fitness related transcripts AC with *F. graminearum*.

Cell-wall degradation is part of *C. rosea* necrotrophic mycoparasitism as shown in interactions with *B. cinerea*

Fig. 7. Representative *Clonostachys rosea* secondary metabolites expressed in monoculture and coculture experiments **A**, Molecular structure, formula, and mass of trichodimerol. **B**, Analysis of variance (ANOVA) box plots of normalized peak area of trichodimerol $[M+H]^+$ pseudomolecular ion (mass error: -0.32 ppm) observed at 5 (before contact) and 7 days (after contact). (Cr = *C. rosea* monoculture; Cr-I = coculture *C. rosea* interaction zone; Fg = *Fusarium graminearum* monoculture; Fg-I = coculture *F. graminearum* interaction zone). **C**, Molecular structure, formula, and mass of TMC-151E. **D**, ANOVA box plots of normalized peak area of TMC-151E $[M+Na]^+$ pseudomolecular ion (mass error: 0.29 ppm) observed at 5 (before contact) and 7 days (after contact).

(Li et al. 2002). *C. rosea* is also predicted to exploit similar mechanisms against *F. graminearum*, although this has not been described to date. To address this hypothesis, putative functions were assigned to significantly upregulated *C. rosea* transcripts at the AC stage to identify those encoding possible cell wall-degradative enzymes (Table 2). Twelve cell-wall degrading and three proteolytic enzyme-encoding homolog transcripts were found to be differentially regulated in *C. rosea* cocultured with *F. graminearum* either BC or AC. A β -galactosidase and β -1,2-glycosyl hydrolase family 61 homologs were significantly expressed at the BC stage, while the α - β hydrolase homolog *BN869_T00014078_1* was up-regulated at both the BC and AC stages but was prominently expressed at the BC stage. Eight glycoside hydrolase homologs were significantly upregulated after the *C. rosea* periphery merged with that of *F. graminearum*. Protease homologs were the second most upregulated *C. rosea* transcripts potentially involved in the *C. rosea* antagonistic activity after colony contact (Table 2). To validate these results, the expression profile of *BN869_T00010437_1* was tested using qPCR and was found to correlate well with the RNA-Seq data (data not shown).

Genes associated with a response to phosphate starvation were up-regulated in *F. graminearum* in the presence of *C. rosea* (Table 3). For example, a *BTA1* homolog was one of

the most prominently induced genes before the *F. graminearum* growing edge merged with that of *C. rosea*. *BTA1* encodes an enzyme known to contribute to the biosynthesis of phosphorus-free betaine lipids (Riekhof et al. 2014). Under conditions of phosphate limitation, betaine lipids can be produced to replace phosphatidylcholine in membranes to conserve phosphate for other essential cell processes. In addition to this, other genes potentially impacted by phosphate limitation were found to be differentially expressed (Table 3). In yeast, the high-affinity phosphate transporter PHO84 and secreted acid phosphatases are induced under phosphate-limiting conditions (Lenburg and O'Shea 1996). Interestingly, the *FGRAMPH1_01G25919* encoding a protein exhibiting 55% amino acid identity to PHO84 was induced in the presence of *C. rosea*. Two genes distantly related to secreted acid phosphatase yeast PHO5 were both upregulated, along with three other genes encoding acid phosphatases. PHO89 is a sodium/phosphate cotransporter, localized to the plasma membrane, with the protein product of *FGRAMPH1_01G08015* sharing 42% amino acid identity with PHO89, and is induced threefold in the presence of *C. rosea*. Yeast GIT1 is a plasma membrane transporter importing glycerol-3-phosphate and phospholipids and is regulated by the availability of phosphate and inositol (Almaguer et al. 2003). *FGRAMPH1_01G13431* is related ($E = 4.6e-77$) to *GIT1* and

Fig. 8. Observed trends in 15-ADON and 15-ADON-3-Glc accumulation in monoculture and coculture experiments **A**, Molecular structure, formula, and mass of 15-ADON. **B**, Analysis of variance (ANOVA) box plots of normalized peak area of 15-ADON $[M+Na]^+$ pseudomolecular ion (mass error: -1.63 ppm) observed at 5 (before contact) and 7 days (after contact). (Cr = *Clonostachys rosea* monoculture; Cr-I = coculture *C. rosea* interaction zone; Fg = *Fusarium graminearum* monoculture; Fg-I = coculture *F. graminearum* interaction zone). **C**, Molecular structure, formula, and mass of 15-ADON-3-Glc. **D**, ANOVA box plots of normalized peak area of 15-ADON-3-Glc $[M+Na]^+$ pseudomolecular ion (mass error: -0.50 ppm) observed at 5 (before contact) and 7 days (after contact).

upregulated fourfold after *C. rosea* confrontation. Phosphate can also be liberated by lipid phosphatidate phosphatase, for which a homolog was induced BC. The induction of these genes involved in nonphosphorus membrane biosynthesis and phosphate transport is similar to expression profiles exhibited during *F. graminearum* infection of maize stalks, which also provide an environment limited in phosphate (Zhai et al. 2016). Expression profiles of *FGRAMPH1_01G01877*, *FGRAMPH1_01G25919*, and *FGRAMPH1_01G15463* detected by RNA-Seq were validated by ddPCR (Supplementary Fig. S2).

The expression of available corresponding *C. rosea* homologs was also evaluated, but only *BN869_T00007330_1* (lipid phosphatidate phosphatase, homolog of *FGRAMPH1_01G12109*) was differentially expressed, with 17-fold (*padj* < 2.47E-25) and sixfold (*padj* < 2.49E-80) induction BC and AC, respectively, during coculture. This suggests that *C. rosea* is not experiencing phosphate limitation to the same extent as *F. graminearum* during the fungal competition.

Differentially regulated *C. rosea* transcripts were explored to identify mechanisms possibly involved in alleviating similar

phosphate starvation and other nutritional scarcity related stress responses. The analysis revealed that, in the confrontation with *F. graminearum*, *C. rosea* enhances its competitiveness, in part, by expanding its carbon source repertoire by induction of the ethanol utilization pathway (Flippi et al. 2003, 2006) as well as redirecting terpene precursors back to the tricarboxylic acid cycle through the mevalonate shunt pathway (Rodríguez et al. 2004). The ethanol utilization pathway (*alc* system) of *Aspergillus nidulans* includes alcohol dehydrogenase (*alcA*), aldehyde dehydrogenase (*aldA*), and a regulatory gene (*alcR*). Putative *C. rosea* *alcS*, *alcA*, *aldA*, and *alcR* transcripts were all up-regulated after the *C. rosea* growing edge contacted that of *F. graminearum* (Supplementary Table S7). The concurrent upregulation of the *alc* pathway with several glycosyl hydrolases after the two fungi make physical contact is also notable. Two transcripts located upstream of *alcA*, putatively encoding acetyl-CoA synthetase and an unknown protein, were also up-regulated. In addition, a cation transporting ATPaseI homolog, methylcrotonyl CoA carboxylase α and β subunits, and two transcripts showing low homology to hydratases residing

Table 2. Expression of selected *Clonostachys rosea* cell wall degradation and proteolysis homeolog transcripts detected by RNA-Seq^a

Transcript ID	Annotation	Fold change (<i>P</i> value)	
		Before contact	After contact
Peptidase			
BN869_T00006507_1	Fungalysin metallopeptidase	1.7 (0.416)	3.5 (3.193E-15)
Protease			
BN869_T00003885_1	Metalloprotease	0.2 (0.36)	16.5 (1.237E-38)
BN869_T00011996_1	Trypsin-like serine protease	1.2 (0.95)	6 (0.0063491)
BN869_T00004333_1	Metalloprotease	1.3 (0.75)	5.1 (5.986E-39)
BN869_T00008115_1	Metalloprotease	0.7 (0.08)	4 (1.387E-32)
Galacto/mano/sidase			
BN869_T00010437_1	β -mannosidase	1.2 (0.95)	22.1 (1.577E-06)
BN869_T00012029_1	Xylosidase arabinofuranosidase	0.4 (0.314)	6.6 (0.0024205)
BN869_T00010326_1	β -galactosidase	11.36 (0.015)	2.72 (0.2527521)
Glucanase			
BN869_T00009871_1	Putative endo- β -1,4-glucanase D	1.2 (0.918)	6.47 (3.758E-10)
Hydrolases_general			
BN869_T00000016_1	Glycoside hydrolase family 28 protein	0.59 (0.852)	6.11 (0.0097954)
BN869_T00000899_1	Glycoside hydrolase family 114 protein	0.98 (0.986)	7.77 (2.472E-07)
BN869_T00003909_1	Related to glycosyl hydrolase	0.31 (0.432)	26.53 (4.119E-19)
BN869_T00004834_1	Glycoside hydrolase family 115 protein	1.15 (0.954)	5.01 (0.0493978)
BN869_T00005806_1	Glycoside hydrolase family 81 protein	1.09 (0.76)	4.99 (2.773E-14)
BN869_T00008474_1	Hydrolase, alpha/beta fold family	0.91 (0.887)	33.92 (3.19E-188)
BN869_T00008724_1	Glycoside hydrolase family 89 protein	0.65 (0.822)	5.32 (8.415E-09)
BN869_T00009923_1	Glycosyl hydrolase family 12	0.59 (0.852)	4.48 (0.0103006)
BN869_T00010029_1	Glycoside hydrolase family 31 protein	0.59 (0.852)	6.55 (0.0112524)
BN869_T00013149_1	Glycoside hydrolase family 61 protein	35.71 (0.0009)	1.23 (0.7037736)
BN869_T00014078_1	α/β hydrolase fold protein	18.52 (3.79E-12)	3.57 (6.782E-25)

^a Fold change values in bold type are significant at *P* value < 0.05, while italicized fold change values are not (*P* value > 0.05).

Table 3. *Fusarium graminearum* genes associated with phosphate starvation are induced before and after being challenged by *Clonostachys rosea*^a

Gene	Annotation	Fold change FDR (<i>P</i> value)	
		Before contact	After contact
<i>FGRAMPH1_01G01877</i>	Betaine lipid synthase (<i>BTA1</i>)	110.03 (0)	1.16 (0.5)
<i>FGRAMPH1_01G08015</i>	Sodium/phosphate cotransporter	2.92 (0)	-1.32 (0.063)
<i>FGRAMPH1_01G12109</i>	Lipid phosphatidate phosphatase	6.80 (8.7E-15)	21.83 (0)
<i>FGRAMPH1_01G12703</i>	Acid phosphatase (<i>PHO5</i> -like)	4.58 (0)	-1.52 (0.007)
<i>FGRAMPH1_01G12793</i>	Acid phosphatase (<i>PHO5</i> -like)	4.03 (4.8E-9)	18.92 (0)
<i>FGRAMPH1_01G13431</i>	Plasma membrane transporter importing glycerol-3-phosphate and phospholipids	4.05 (0)	1.43 (0.004)
<i>FGRAMPH1_01G15463</i>	Acid phosphatase	32.98 (0)	-2.02 (3.7E-5)
<i>FGRAMPH1_01G19107</i>	Acid phosphatase	8.99 (0)	1.02 (0.99)
<i>FGRAMPH1_01G25367</i>	Acid phosphatase	12.40 (0)	-2.25 (6.7E-14)
<i>FGRAMPH1_01G25919</i>	High-affinity phosphate transporter	16.84 (0)	-1.31 (0.034)
<i>FGRAMPH1_01G04877</i>	Lipid phosphate phosphatase 2	1.19 (0.27)	7.18 (0)
<i>FGRAMPH1_01G22711</i>	Alkaline phosphatase	115.39 (0)	-1.84 (0.018)

^a Fold change values in bold type are significant at FDR *P* value < 0.05, while italicized fold change values are not (*P* value > 0.05). FDR = false discovery rate.

downstream of the *alc* pathway homolog transcripts were also up-regulated. Methylcrotonyl CoA carboxylase α and β sub-units along with *alcA*, *aldA*, and hydratases are involved

converting the isoprenoid pathway general precursors into acetyl-CoA through the mevalonate shunt pathway (Brady et al. 1982).

Table 4. List of upregulated MFS type transporter homolog transcripts at *P* value < 0.05 and their classification^a

No.	Transcript ID	Before contact		After contact		MFS Family
		Fold change	<i>P</i> value	Fold change	<i>P</i> value	
1	BN869_T00004802_1	8.06	0.00093	1.20	0.00101	2.A.1.1 (the sugar porter family)
2	BN869_T00009898_1*	5.85	4E-20	5.89	4E-121	
3	BN869_T00000518_1	4.37	4.2E-05	0.75	1.6E-18	
4	BN869_T00013994_1*	3.16	1.1E-11	2.63	7.2E-67	
5	BN869_T00002616_1	1.60	3.4E-12	2.73	0	
6	BN869_T00011473_1	1.08	0.91019	3.07	0.04738	
7	BN869_T00010393_1	0.85	0.85195	7.86	1.2E-05	
8	BN869_T00002201_1	0.79	0.20142	4.24	6.3E-80	
9	BN869_T00010022_1	0.79	0.53635	2.56	1.6E-18	
10	BN869_T00000891_1	0.77	0.35862	5.96	3.9E-54	
11	BN869_T00005632_1	0.77	0.00727	2.49	0	
12	BN869_T00012137_1	0.67	0.40332	2.50	3E-11	
13	BN869_T00011842_1	0.56	0.48441	6.41	7.1E-05	
14	BN869_T00013528_1	0.53	0.00892	2.70	4.3E-20	
15	BN869_T00010377_1	0.39	0.00012	3.28	2.8E-27	
16	BN869_T00008572_1	0.38	0.02242	2.68	1E-05	
17	BN869_T00012857_1	0.15	1.4E-23	2.54	1E-125	
18	BN869_T00005309_1	2.60	0.02687	0.19	4.7E-08	2.A.1.12.2 (the lactate/pyruvate:H ⁺ symporter)
19	BN869_T00005193_1*	2.49	0.00319	2.17	2.7E-15	
20	BN869_T00000934_1	2.46	1.2E-09	1.56	0.00031	2.A.1.14 (the anion:cation symporter family)
21	BN869_T00004864_1	1.30	0.70339	3.47	0.01532	
22	BN869_T00013019_1	5.99	0.19126	0.26	1.1E-05	
23	BN869_T00006489_1	4.03	8.9E-07	0.40	0.00159	
24	BN869_T00002489_1	2.58	2.5E-05	0.74	0.08715	
25	BN869_T00012163_1	1.16	0.80486	5.11	0.00066	
26	BN869_T00008448_1	0.60	0.4373	2.49	0.00037	
27	BN869_T00013493_1	0.56	0.36767	2.93	0.00136	
28	BN869_T00001004_1	0.49	0.50626	5.49	0.00164	
29	BN869_T00009316_1	0.39	0.05858	2.61	6.1E-07	
30	BN869_T00012452_1	0.33	8.3E-06	2.75	3.5E-09	
31	BN869_T00007109_1	0.29	0.04039	2.56	9E-20	
32	BN869_T00008582_1	0.26	0.0004	2.57	1E-07	
33	BN869_T00006919_1	0.25	0.33948	12.63	2.2E-06	
34	BN869_T00009001_1	0.25	4.4E-06	3.52	7.1E-10	
35	BN869_T00004349_1	0.22	0.04772	4.65	2.3E-09	
36	BN869_T00000978_1	0.16	0.15304	3.80	0.04478	2.A.1.2 (the drug:H ⁺ antiporter-1 [12 spanner] [DHA1] family)
37	BN869_T00007234_1*	125.00	8E-103	56.35	1.4E-92	
38	BN869_T00000794_1	4.22	2.2E-17	0.49	3.6E-52	
39	BN869_T00004654_1	2.90	4.3E-17	0.54	1.1E-45	
40	BN869_T00001547_1	2.48	1.9E-10	0.47	1.8E-74	
41	BN869_T00014038_1	3.23	0.1365	2.60	0.02074	
42	BN869_T00008955_1	2.71	0.13085	2.01	4.9E-05	
43	BN869_T00005769_1	0.98	0.99265	2.53	0.02697	2.A.1.3 (the drug:H ⁺ antiporter-2 [14 spanner] [DHA2]—most toxin efflux MFS belong to this family)
44	BN869_T00005588_1	0.52	0.53732	2.87	0.00474	
45	BN869_T00002203_1	0.44	0.00023	2.86	4.5E-31	
46	BN869_T00007594_1	14.29	2.4E-31	0.61	2.3E-10	
47	BN869_T00013211_1	12.99	9.5E-05	1.46	0.31936	
48	BN869_T00013338_1	3.83	0.01993	0.49	2E-212	
49	BN869_T00010959_1	2.74	1.9E-17	0.10	8E-307	
50	BN869_T00005590_1	2.72	9.5E-08	0.85	0.33959	2.A.1.7 (Uncharacterized protein)
51	BN869_T00009641_1	2.68	0.04434	0.51	2E-06	
52	BN869_T00009232_1	2.65	1.3E-08	0.97	0.74365	
53	BN869_T00006965_1	2.59	2.5E-06	0.78	0.11555	
54	BN869_T00008098_1	1.04	0.92697	2.54	3.9E-09	
55	BN869_T00011832_1	0.91	0.89723	3.29	4.5E-33	
56	BN869_T00005651_1	0.84	0.77451	3.07	1.8E-17	
57	BN869_T00007791_1	0.16	0.28372	3.69	0.01659	
58	BN869_T00002816_1	4.93	6.7E-14	0.71	3.8E-07	
59	BN869_T00010994_1	4.31	0.01481	0.77	0.1215	
60	BN869_T00012492_1	0.87	0.90491	3.87	8.3E-07	
61	BN869_T00006371_1	0.73	0.16305	2.71	1.1E-18	
62	BN869_T00005531_1	1.11	0.45855	4.72	5E-151	Unknown family
63	BN869_T00006259_1	1.61	6.1E-10	2.50	7E-156	

^a Significant expression values are given in bold. Asterisks (*) indicate transcripts upregulated both before and after contact.

Differential regulation

of *C. rosea* MFS transporter homolog transcripts.

MFS are ubiquitous membrane transporter proteins involved in substrate translocation across cell membranes (Gbelska et al. 2006). A total of 63 unique *C. rosea* MFS homolog transcripts were up-regulated at one or both BC and AC stages, of which 20 were exclusively expressed BC and 39 AC. Four transcripts were up-regulated at both stages. In a previous study, MFS homolog transcripts were among the most upregulated transcripts 24 h after *C. rosea* IK726 mycelium made contact with that of *F. graminearum* PH-1 (Nygren et al. 2018). In our study, of the 22 MFS homologs upregulated in IK726 interacting with PH-1, only six were significantly upregulated in *C. rosea* (strain ACM941) cocultured with *F. graminearum* (strain GZ3639) (Supplementary Table S8). Of the six transcripts in common, *BN869_T00007234_1* was up-regulated both BC and AC, while others were up-regulated only AC (Table 4). This transcript was the most up-regulated transcript in IK726 cocultured with PH-1, and a homolog of this was the second most up-regulated transcript in *C. rosea* (strain ACM941) interacting with *F. graminearum* (strain GZ3639). As shown in Figure 2, transcript *BN869_T00007234_1* was up-regulated as part of a gene cluster, which included transcripts showing homology to NADPH dehydrogenase, hydrolase, acetamidase, and a transcription factor.

According to the Transporter Classification Database (Saier et al. 2016), proteins included in the MFS family were classified into different subfamilies based on their sequence homology and conserved function. The main upregulated MFS proteins in *C. rosea* belong to subfamily 2.A.1.1—sugar transporters, 2.A.1.2—the drug:H⁺ antiporter-1 (DHA1), 2.A.1.3—the drug:H⁺ antiporter-2 (DHA2), 2.A.1.13—monocarboxylate transporters, and 2.A.1.14—the anion:cation symporters (ACSSs). Upregulated *C. rosea* MFS proteins classified as sugar transporters, DHA2, and ACSSs were either up-regulated at BC or AC stages, compared with the other subfamilies. For example, 13 of 17 transcripts belonging to the family of the sugar porter subfamily were exclusively up-regulated in *C. rosea* AC with *F. graminearum*, while two were up-regulated both BC and AC (Table 4). In addition, 12 of the 15 symporters that use organic anions as substrates, including cell-wall degradation products, were up-regulated AC. It is notable that the expression of these transcripts correlated well with the expression of CWDE homolog transcripts (Table 2). This, coupled with the fact that phosphate-starvation response genes were not significantly upregulated in *C. rosea* as opposed to *F. graminearum* (Table 3) indicates the competitive advantage of *C. rosea* over *F. graminearum* in this limited resource environment. Together these results raise another new hypothesis, wherein *C. rosea* has increased competitive fitness in the later stage of the interaction, including uptake of nutrients released during degradation of *F. graminearum*.

In contrast, eight of the 12 upregulated transcripts of the DHA2 subfamily were up-regulated BC (Table 4). The DHA2 subfamily members are generally recognized for their mycotoxin efflux transport properties (Gbelska et al. 2006); therefore, the fact that their expression corresponds with the expression of secondary metabolite-related gene clusters (Fig. 5) is notable. An approximately even number of transcripts belonging to the DHA1 subfamily were also upregulated both BC and AC (three and five transcripts, respectively).

DISCUSSION

Previously a concentration-dependent growth inhibition of *F. graminearum* GZ3639 by *C. rosea* ACM941 liquid culture filtrate was observed (Demissie et al. 2018), suggesting that

C. rosea secretes secondary metabolites inhibitory to the growth of *F. graminearum*. This work corroborated previous reports (Dubey et al. 2014; Rodríguez et al. 2011) and comparative genomic study-based predictions (Karlsson et al. 2015, 2017). As *C. rosea* is a necrotrophic mycoparasite that can overgrow its prey, secretion of CWDEs was also anticipated. Notably, microscopic studies have revealed that the destruction of *B. cinerea* by *C. rosea* is preceded by *C. rosea* coiling around or physically penetrating *B. cinerea* (Li et al. 2002; Yu and Sutton 1997). Together, these reports imply that *C. rosea* utilizes both secreted secondary metabolites and CWDEs toward enabling its antagonistic activity. Consistent with this, the biochemical basis of *C. rosea* mycoparasitism against *Botrytis allii* was reported to involve both toxin production and expression of CWDEs (Pachenari and Dix 1980). However, it is notable that signaling pathways activated in response to secreted molecules differ from those induced upon physical contact (Luo et al. 2017). This raises a hypothesis that responses from *C. rosea* to *F. graminearum* secreted signaling molecules differ from those arising from physical contact. Toward defining the specific contributions of the various molecular networks from a temporal perspective, the transcriptomic and exometabolomic responses of *C. rosea* cocultured with *F. graminearum* were compared with their monoculture counterparts before and after interfungal contact.

Interestingly, only 41 transcripts were observed to be differentially regulated in *C. rosea* IK726 interacting with *F. graminearum* PH-1 (Nygren et al. 2018). This is in contrast, to the 2,082 transcripts that were differentially regulated when *C. rosea* ACM941 was cocultured with *F. graminearum* GZ3639 at a similar interaction stage, described herein. In addition to inherent differences between the different strains and analysis methods used, it is also likely that the use of the minimal media Czapek Dox in the confrontation between IK726 and PH-1 may have influenced IK726 transcriptional response. In particular, secondary metabolite production-related gene clusters are generally activated by nutrient stress (Keller 2019), potentially masking their differential expression. This was addressed herein by the use of nutrient-rich media to minimize external stress in the interaction responses. As well, *C. rosea* was grown for 10 days before introducing *F. graminearum*, as compared with the 5 days afforded to IK726. Prior incubation of *C. rosea* was required not only to account for its slow-growing nature but also to mimic its in-situ biocontrol activity. *B. cinerea* control by *C. rosea* in raspberry tissue was superior when *C. rosea* was applied prior to pathogen inoculation and was weakened significantly when inoculated at the time of pathogen inoculation or later (Yu and Sutton 1997). The slow-growing habit of *C. rosea* could also be the result of its conspicuous secondary metabolite biosynthetic ability when competing for carbon sources (Fig. 6). Consistent with this, *nrpS1* deletion mutants of IK726 were found to grow faster with more abundant conidiation, as compared with their wild-type counterpart (Iqbal et al. 2019).

F. graminearum secreted molecules induce secondary metabolite biosynthesis in *C. rosea*.

C. rosea and other similar biocontrol agents can use their chemical arsenal both to mediate beneficial effects on plants and to antagonize pathogenic organisms like *Fusarium* spp. However, this knowledge has been largely derived from gene expression profiling experiments or comparative genomics analysis of genes putatively encoding proteins involved in secondary metabolite biosynthesis rather than from direct investigations of the metabolites synthesized during the interaction (Demissie et al. 2018; Dubey et al. 2014; Karlsson et al. 2015). Consistent with observations from previous genetic

studies, an accumulation of significantly greater amounts of distinct secondary metabolites were reported in the confrontation zone along the periphery of both *C. rosea* and *F. graminearum* during coculture (as compared with monocultures). Although most of the *C. rosea* extracellular metabolites produced in response to *F. graminearum* were not annotated, the polyketides trichodimerol/bisorbicillinoid analogs (Fig. 7A, B) and TMC-151-related metabolites (Fig. 7C, D) were among those produced in significantly higher amounts. Polyketides include a diverse array of natural products synthesized by the modular enzymes called PKSs and are hypothesized to play roles in *C. rosea* biocontrol activity. PKSs are some of the most enriched gene families in the *C. rosea* genome (Karlsson et al. 2015, 2017) and many polyketides have demonstrated antifungal properties (Kohn et al. 1999; Okuda et al. 2000; Zhai et al. 2016). For example, bisorbicillinoids are biosynthesized by gene clusters containing two PKSs, reducing and nonreducing PKS (Guzmán-Chávez et al. 2017), and have been isolated from biocontrol agents like *Trichoderma citrinoviride* (Evidente et al. 2009) and *C. rosea* YRS-06 (Zhai et al. 2016). Consistent with this, a putative *C. rosea* sorbicillinoid-type gene cluster (identified by Fungi-SMASH) was found to be slightly upregulated BC (Table 1), consistent with the detection of bisorbicillinoid accumulation in the *C. rosea* colony periphery during coculture (Fig. 7A and B).

Exhibiting strong antimicrobial activity, TMC-151 related metabolites are thus far exclusive to *C. rosea* (Ju et al. 2007; Zhai et al. 2016). Certain TMC-151 and bisorbicillinoid analogs exhibited superior antibacterial activity compared with levofloxacin, erythromycin, streptomycin, ampicillin, and tetracycline against *Escherichia coli*, *Clostridium perfringens*, and *Bacillus subtilis* (Zhai et al. 2016). In the same study, culture broths of a *C. rosea* strain (YRS-06) were also found to have antifungal activity against several plant pathogens; however, a link between TMC-151 and bisorbicillinoid analogs and observed antifungal activities has yet to be tested. In this current study, the putative TMC-151 gene cluster reported previously from *C. rosea* ACM941 (Demissie et al. 2018) was not found to be upregulated BC or AC from *C. rosea* cocultured colonies. One possible explanation of this variance is the time of sampling. In the previous study, upregulation of the proposed TMC-151 biosynthetic gene cluster was detected only 3 days after spore inoculation into liquid broth containing *F. graminearum* secretome metabolites (Demissie et al. 2018). Thus, most likely, sampling for the BC timepoint on day 5 in this current study was too late to detect the transient induction of the previously proposed cluster. While the possibility that this is not, in fact, a TMC-151 biosynthetic gene cluster cannot be strictly eliminated, it is notable that no other suitable upregulated gene cluster presents itself as an alternative. A lack of correlation between observed transcript expression of a gene cluster and that of the resulting product accumulating is not uncommon in fungi (Okada and Seyedsayamdost 2017).

When *F. graminearum* was confronted by *C. rosea*, the expression of 15 secondary metabolite gene clusters was significantly affected. *TRI* gene expression and trichothecene biosynthesis increased (Fig. 3) as did expression of genes responsible for ZEA and aurofusarin biosynthesis, while expression of genes responsible for fusarielin and fusarin biosynthesis decreased (Supplementary Table S6). This is consistent with a previous report that showed that upregulation of trichothecenes was accompanied by a decrease in other types of secondary metabolites derived from common precursors like acetyl CoA (Atanasova-Penichon et al. 2018). While many of the end products of gene clusters induced prior to contact exhibit antifungal activity (Medentsev et al. 1993; Utermark and Karlovsky 2007), the upregulation of these gene

clusters was not maintained AC. Many of the differentially expressed gene clusters remain uncharacterized and some of the tailoring enzymes (e.g., cytochrome P450s, oxidoreductases, and glucosyltransferases) may be induced as part of a detoxification process.

***C. rosea* shifts its antagonistic mode to cell-wall degradation after physical contact.**

Transcription of *C. rosea* genes encoding various CWDEs (Table 2) support the hypothesis that CWDEs play a role in the necrotrophic and mycoparasitic nature of *C. rosea*. In this regard, enzymatic activity of β -(1,3)-glucanases and chitinases by *C. rosea* have been experimentally demonstrated (Pachenari and Dix 1980); however, to date only an endochitinase from *C. rosea* 67-1 has been shown to play a role in its antagonism as a mycoparasite (Sun et al. 2017). In contrast, deletion of a pectate lyase gene that was enriched in the *C. rosea* IK726 genome did not affect its antagonism against *Fusarium* spp. (Atanasova et al. 2018), perhaps due to the redundancy of CWDEs. In agreement with the findings reported by Pachenari and Dix (1980), the present study has shown that secondary metabolism-related transcripts were up-regulated BC between *C. rosea* and *F. graminearum*, while proteolysis and cell wall degradation-related transcripts were mostly up-regulated AC (Table 2). These results imply that the *C. rosea* lifestyle transitions from secondary metabolite-mediated antibiosis to prey destruction BC and AC with *F. graminearum*, respectively. In particular, proteolysis-related enzymes like metalloproteases and peptidases, and CWDEs in the family of glucosyl hydrolases were up-regulated AC. Similar types of proteolytic CWDEs have been described to play a role during the necrotrophic stage of *Colletotrichum* plant pathogens (Gan et al. 2013; O'Connell et al. 2012).

Glycosylation plays a central role in *C. rosea* tolerance of 15-ADON.

Tolerance of *C. rosea* ACM941 and other strains to various fungal toxins has long been attributed to membrane transporters, largely based on one or both the relatively high number of ABC-transporter genes in their genome and their increased transcriptional activity upon exposure to toxigenic fungi. Notably, *C. rosea* ABC transporter genes are reported to be upregulated upon exposure to fungal mycotoxins (such as the *Fusarium* mycotoxin ZEA), bacterial toxins from *Pseudomonas chlororaphis*, or fungicides like Boscalid (Kamou et al. 2016). However, the exact role and the extent to which ABC transporters contribute to toxin tolerance has yet to be elucidated. For example, the yeast ABC transporter *pdr5* is up-regulated upon exposure to DON and its deletion or mutation of key residues in its active site reduced tolerance for DON in vivo (Gunter et al. 2016). However, the role of *pdr5* in direct transport of DON has not been established. Similarly, despite *C. rosea* IK726 *pdr5* transcriptional activity and deletion assay results (Dubey et al. 2014; Kosawang et al. 2014), ZEA is detoxified by ZHD101 enzyme (Takahashi-Ando et al. 2002). In agreement with these reports, exometabolomic analysis established that 15-ADON was transformed to 15-ADON-3-Glc, accumulating in the agar proximal to *C. rosea*, suggesting a role for enzymatic detoxification and possibly membrane transport in the tolerance process. The detoxification of 15-ADON in *C. rosea* is achieved (wholly or, at least, in part) through glycosylation, possibly mediated by a glucosyltransferase enzyme, and is likely exported by a membrane transport system composed of either MFS proteins or ABC transporters. In support of this hypothesis, two ABC transporter homologs and several MFS proteins with homology to those implicated in xenobiotic tolerance were up-regulated in

C. rosea (Table 4). That said, the possibility that the detoxifying enzyme is itself secreted, eliminating the need for small molecular transport, cannot be strictly eliminated at this time.

Glycosylation of DON by partially resistant plants (Li et al. 2015, 2017; Steiner et al. 2017) and *Trichoderma* spp. has been reported previously (Tian et al. 2016a and b, 2018). However, this is the first report involving direct glycosylation of 15-ADON. Notably, a recent report concluded that *C. rosea* stimulates the ability of maize to elicit the glycosylation of DON (Abdallah et al. 2018). However, this current study now provides substantial evidence that *C. rosea* is itself capable of 15-ADON glycosylation, and thus, the possibility that *C. rosea* may have contributed to the glycosylation reaction reported for maize plants challenged with *F. graminearum* and *C. rosea* must be considered (Abdallah et al. 2018).

MFS genes are the most common upregulated genes reported in several fungal-fungal interaction studies. In this study, 43 of the 67 (63 plus four duplicates) differentially regulated *C. rosea* MFS genes were up-regulated AC with *F. graminearum* mycelia. Interestingly, these MFS genes were mainly dominated by those classified under sugar transporters family (2.A.1.1) and the ACS family (2.A.1.14), with 15 and 12 members, respectively. Representation in the symporter family was largely dominated by the high-affinity nicotinate permeases (2.A.1.14.11), while the galacturonic acid uptake porter (2.A.1.1.119) dominated the sugar transporter family. In general, sugar transporters and ACS family MFS-encoding genes are enriched in endophytic microbe genomes, because they are involved in nutrient acquisition from their host. For example, *Pestalotiopsis fici* (a pathogenic plant endophyte) had four times more ACS family genes compared with other studied genomes (Wang et al. 2015). Notably, the expression profile of these transcripts correlated well with the upregulation of cell wall degradative enzyme-encoding transcripts. In contrast, the MFS family 2.A.1.3 (xenobiotic secretion) expression profile correlates well with the upregulation of secondary metabolism-related gene clusters, as described above. Taken together, these results emphasize that the *C. rosea* transcriptomic response profile transitions from one dominated by secondary metabolism during the BC stage, to cell-wall degradation and nutrient acquisition AC in its confrontation with *F. graminearum*. Fungal lifestyle transitions depending on the available nutrient source is a well-established phenomenon. Indeed, the lifestyle of *Colletotrichum* spp. (plant pathogens) transitions from secondary metabolism biosynthesis during colonization to cell wall degradative enzyme-encoding genes at the necrotrophic stage (Gan et al. 2013; O'Connell et al. 2012).

Finally, the observed *C. rosea* transition from secondary metabolism BC to necrotrophism AC with *F. graminearum* was also accompanied by upregulation of gene clusters likely to play a role in expansion of its carbon source utilization. One such example is the upregulation of the ethanol utilization pathway (Flipphi et al. 2003). This pathway was first described in *Aspergillus nidulans*, in which its expression enabled the fungus to utilize a wide range of organic compounds as sole sources of carbon and nitrogen. Upregulation of the putative alcS gene cluster in *C. rosea* occurred upon contact with *F. graminearum* or coincided with *C. rosea* moving to a resource depleted area of the agar plate, or both (Supplementary Table S7), heightening the plausibility that *C. rosea* expresses the alcS pathway to catabolize an alternative carbon source.

In conclusion, the results highlighted here demonstrate that the antagonistic response of *C. rosea* (strain ACM941) toward *F. graminearum* (strain GZ3639) is initiated before the two organisms come into direct contact and includes upregulation of secondary metabolism-related genes and metabolite

production. While trichothecenes are the main defense arsenal produced by *F. graminearum*, *C. rosea* produces a multitude of different metabolites mainly representing polyketides—trichodimerol and related analogs of TMC-151. The abundance of secondary metabolism-related transcripts returns to the basal level while *C. rosea* transitions to activating the transcription of genes encoding cell wall-degradation enzymes upon actual contact with *F. graminearum*. The majority of cell wall degradation-related transcripts were not differentially regulated BC. Notably, findings herein also demonstrate that one method of *C. rosea* tolerance of *F. graminearum* trichothecenes is via glycosylation to 15-ADON-3-Glc. While efforts are ongoing to identify the mechanistic components underlying this, it must be emphasized that this is the first report to describe a trichothecene detoxification functionality for *C. rosea*. Overall, a number of signature molecular mechanisms responsible for the *C. rosea* antagonistic activity against *F. graminearum* as well as its mycotoxin tolerance were identified in this study, yielding several new testable hypotheses toward understanding the basis of naturally occurring biocontrol with potential applications for agronomic development in the future.

MATERIALS AND METHODS

Fungal strains and culture condition.

C. rosea ACM941 (American Type Culture Collection 4447), provided by A. Xue (Ottawa Research and Development Centre, Agriculture and Agri-Food Canada [AAFC]) with permission from Adjuvant Plus Inc, was grown at 25°C for 10 to 12 days and was maintained on potato dextrose broth (Bioshop, Canada) containing 15 g of Bacto agar per liter (Difco Laboratories, Detroit) (PDA) plates at 4°C for short-term storage. *F. graminearum* GZ3639 (Proctor et al. 1995), obtained from S. McCormick (United States Department of Agriculture, Peoria), was grown for 5 to 6 days and was maintained as per *C. rosea*. For long-term storage, agar plugs (five or six) taken from the growing edge of each fungal colony were used to inoculate carboxymethylcellulose (CMC) media (per liter, 1 g of NH₄NO₃, 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 1.0 g of yeast extract, and 15 g of CMC) and were grown for 10 days at 25°C. Following mycelial removal by filtering the media through four or five layers of cheesecloth, macroconidia were pelleted by centrifugation at 4,000 × g for 20 min at 4°C and were washed twice, using sterile water. Macroconidia were resuspended in 0.5 to 1 ml of sterile water, and the concentration was determined by counting using a hemocytometer. The final concentration of macroconidia in this stock was adjusted to 10⁷ ml⁻¹ in sterile water containing 20% glycerol, was flash-frozen in liquid nitrogen, and was stored at -80°C. The 15-ADON standard was purchased from Cayman Chemical, the ZEA standard was purchased from Sigma, and the gramillan standard was obtained from B. Blackwell (AAFC).

An agar plug of the actively growing edge of *C. rosea* mycelia (harvested using the wide-end of a 1-ml sterile pipette tip for consistency) was inoculated on PDA plates approximately 1 cm away from the edge of the plate and was grown in the dark at 25 to 28°C. After growing *C. rosea* alone for 10 days (or after the mycelium covered approximately one third of the plate), agar plug of actively growing *F. graminearum* edge was inoculated (approximately 1 cm away from the side of the plate) on the opposite side of the plate from where *C. rosea* was inoculated. Controls for each strain were inoculated similarly but were grown as monocultures. Early inoculation of *C. rosea* was necessary to compensate for the slow-growing nature of the fungus, allowing for sufficient mycelia to accumulate for RNA extraction (discussed below). The experiment was set up as a

7-day time course (postinoculation of *F. graminearum*). For metabolomics analyses, sampling occurred on days 3, 4, 5, and 7, for which six agar plugs were excised, using a core borer from the agar adjacent to the colony periphery (of both *C. rosea* and *F. graminearum* colonies), and were stored in scintillation vials at -80°C , until metabolites were extracted. The experiment was done in six replicates, with individual plates for each of the different sampling timepoints. For transcriptomic analyses, samples were collected first, when the growing edges of *C. rosea* and *F. graminearum* in confrontation plates were approximately 1 cm apart (day 5), referred to as BC, and second, 12 to 16 h after the growing edges of *C. rosea* and *F. graminearum* colonies in confrontation plates made contact (with no visible gap in between; day 7), referred to as AC (Supplementary Fig. S1). *C. rosea* and *F. graminearum* colony mycelia were scraped from both control and coculture plates for RNA extraction, following the excision of the agar plugs for metabolomics analysis as described above (Supplementary Fig. S1). Mycelia were flash-frozen in liquid nitrogen and were used for total RNA isolation, either immediately or after a brief storage in a -80°C freezer.

Secreted secondary metabolite profiling and metabolomic analysis.

Each sample was extracted with 10 ml of ethyl acetate for 1.5 h, gently shaking at 150 rpm, and the resulting extracts were evaporated to dryness. Extracts were subsequently reconstituted in methanol (MeOH) to a concentration of $500\text{ }\mu\text{g ml}^{-1}$ and were chemically profiled in a randomized order, using a Thermo Ultimate 3000 UPLC coupled to a Thermo LTQ Orbitrap XL HRMS. Chromatography was performed on a Phenomenex C₁₈ Kinetex column (50 mm $\times$ 2.1 mm internal diameter, 1.7 μm) with a flow rate of 0.35 ml min^{-1} , running a gradient of H₂O (+ 0.1% formic acid) and acetonitrile (ACN) (+ 0.1% formic acid), starting at 5% CAN, increasing to 95% ACN by 4.5 mins, and held at 95% ACN until 8.0 min, returning to 5% ACN by 9 min, and holding, to allow the column to equilibrate to starting conditions by 10 mins. The HRMS was operated in ESI⁺ mode (with a mass to charge ratio [m/z] of 100 to 2,000 m/z), using the following parameters: sheath gas (40), auxiliary gas (5), sweep gas (2), spray voltage (4.2 kV), capillary temperature (320°C), capillary voltage (35 V), and tube lens (100 V).

All .RAW data files, including samples, MeOH blanks (run after every sixth sample), and medium controls, were processed using MZMine v2.29 (Cell Unit, Okinawa Institute of Science and Technology). For data preprocessing, mass detection was carried out with a noise cut-off level of (5.0 E^5) using the FWHM (full width at half maximum) algorithm and deconvoluted with a chromatographic threshold of 35%, a retention time (RT) range minimum of 0.05 min, a minimum relative height of 35.0%, and a minimum ratio of peak top/edge of 1.2. Data were then cleared of isotopes and were aligned and converted into a data matrix of discriminate variables (based on RT and m/z) based on peak area measurements. Peaks were aligned using the Join Alignment function (with a m/z tolerance of 5.0 ppm and a RT tolerance of 0.1 min with a 20:10 weight for m/z versus RT). Gaps in the data set where variables fell below the noise limit detection threshold were backfilled using a gap-filling algorithm (using a m/z tolerance of 5 ppm and RT tolerance of 0.05 min). Peak area values were normalized by dividing by the total ion current for each sample. Multivariate and univariate statistical analysis was performed in the R environment using the muma package and Metaboanalyst.

A database of secondary metabolites previously reported from *C. rosea* and *F. graminearum* was compiled from a comprehensive literature search to generate a mass list of expected pseudomolecular ions (protonated mass, adducts,

neutral losses, fragments) and was used for signal annotation. For confirmation of signal annotations, in-source fragmentation and MS/MS experiments were carried out along with comparison of UV-absorbance spectra to literature values and, when possible, were compared with standards (i.e., 15-ADON and other associated trichothecenes, gramillins, culmorin, and zearalanone).

Confirmation of 15-ADON glycosylation by *C. rosea*.

To confirm 15-ADON glycosylation ability, *C. rosea* was grown on PDA plates for 10 days and triplicate sterile filter discs (dosed with 50 μl of differing concentrations of 15-ADON [125, 250, or $500\text{ }\mu\text{g ml}^{-1}$] or MeOH vehicle control) were placed approximately 1.5 cm away from the fungal edge. After 6 days of incubation, the agar on either side of the filter discs was excised and extracted in ethyl acetate for 2 h, was dried down, and was reconstituted in 1 ml of MeOH for UPLC-HRMS analysis. The experiment was performed in triplicate.

Transcriptome profiling.

Total RNA extraction and RNA-Seq. Freeze-dried mycelium (both *C. rosea* and *F. graminearum*) was ground to a fine powder and was homogenized in 1 ml of TRIzol reagent (Thermo Fisher Scientific). The InviTrap Spin Universal RNA mini kit (Stratag Molecular) was used to purify total RNA (free of genomic DNA) from TRIzol aqueous phase, as per manufacturer protocol; the RNA concentration and purity was subsequently determined using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific). RNA-Seq libraries were prepared using TruSeq Stranded RNALT kit and sequenced on an Illumina HiSeq 2500 platform according to the manufacturer guidelines (Illumina).

Differential gene expression analysis and validation.

C. rosea. Trimmomatic software v0.36.4 was used to trim low-quality short reads and adapter and other Illumina-specific sequences with the following modifications to the default settings: leading low-quality cutoff was 17, sliding window was 5 bp with a minimum average quality score of 20, and read-length cutoff was 60 bp instead of 36 bp (Bolger et al. 2014). Paired-end *C. rosea* ACM941 reads were cleaned and mapped to *C. rosea* IK726 putative transcripts (accessed on Sept 14, 2017) using the read mapper software STAR version 2.5.1 b (Dobin et al. 2013). Raw read counts per gene were extracted with the software HTSeq-count v0.6.0 (Anders et al. 2015), following procedures described previously (Perlea et al. 2016, 2015). The DESeq2 based SARTools (v1.5.1) pipeline was adopted for differential analysis of mapped *C. rosea* ACM941 RNA-Seq count data—coculture relative to monoculture (Varet et al. 2016). A Benjamini-Hochberg *P* value adjustment was performed (Benjamini and Hochberg 1995; Benjamini and Yekutieli 2001) and the false discovery rate (FDR) was set at $P < 0.05$. For comparison, high-quality *C. rosea* ACM941 reads were mapped against the *C. rosea* CBS12511 genome and annotated genes (unpublished data made available by JGI) as described above. Coding sequences of each gene were subtracted from the reference genome sequence with the tools bedtools v2.4.19 (Neph et al. 2012) and bedtools v2.26.0 (Quinlan and Hall 2010), and raw read counts per gene extraction and subsequent differential gene expression analysis was performed as described above. Blastx-fast (E value $< 10^{-3}$) module of BLAST2Go was used to align translated *C. rosea* transcripts (upregulated >2.5 -fold BC and AC) against the National Center for Biotechnology Information nonredundant protein library database (last accessed April 20, 2019) to improve annotation coverage. After annotation, GO terms and enzyme codes associated with each transcript were retrieved, using default parameters to determine their biological role and for graphical presentations.

To validate RNA-Seq gene expression results, primers (Supplementary Table S2) were designed using the IDT primer quest software targeting 190- to 200-bp fragments. Complementary DNA (cDNA) was synthesized from total RNA stored at -80°C (remaining from samples shipped for sequencing) using the 5 $\times$ All-In-One RT MasterMix kit (ABM, Inc.). The relative transcript abundance of the selected genes was assessed by IQ5 multicolor real-time PCR detection system (Bio-Rad), using the EvaGreen Express 2 $\times$ qPCR MasterMix (ABM, Inc.) along with approximately 150 ng of cDNA template and 500 nM of each of the primers in a 20- μl reaction volume. The following program was used for reverse transcription-qPCR: 95°C for 1 min followed by 40 cycles of 5 s at 95°C and 15 s at 59°C . Normalized relative expression values ($\Delta\Delta\text{C}_T$) of the selected candidates were calculated using the formula $2^{-\Delta\Delta\text{C}_T}$ (Livak and Schmittgen 2001), using *EF1 α* and *β -actin* as reference genes (Supplementary Table S2). Serial dilutions of cDNA samples were used to develop standard curves for primer sets to confirm their efficiency according to the Livak relative-gene expression quantification method.

F. graminearum. The *F. graminearum* RNA-Seq data were analyzed using CLC Genomics workbench version 11.0.1 (Qiagen Corp.). Raw data were trimmed based on default quality scores that were determined by the base caller error probability level ($P < 0.01$). To estimate the expression levels, high-quality RNA sequences were aligned to the *F. graminearum* RR1.36 genome annotated with genes and transcripts (King et al. 2015). Read alignment was performed using high-stringency criteria to eliminate *C. rosea* transcripts: similarity fraction = 0.95 and length fraction = 0.8, mismatch cost = 2, deletion and insertion costs = 3, and maximum number of hits per read = 10. Gene-expression levels were estimated as transcripts per million (TPM) (Li et al. 2010) which was calculated as: $\text{TPM} = (\text{RPKM} \times 10^6) / \sum \text{RPKM}$, where the sum is over the RPKM (reads per kilobase of exon model per million mapped reads) values of all genes or transcripts. RPKM (Mortazavi et al. 2008) was calculated following the formula: $\text{RPKM} = \text{total exon reads} / [\text{mapped reads (millions)} \times \text{exon length (KB)}]$.

An empirical analysis of differential gene expression or the ‘exact test’ according to (Robinson and Smyth 2008) was implemented to compare monocultured versus cocultured fungal inoculations. The exact test, a package also incorporated in EdgeR Bioconductor (Robinson et al. 2010), is similar to Fisher’s exact test but also accounts for overdispersion caused by biological variability. Transcripts were considered as significantly differentially expressed when fold change was ≥ 4.0 , FDR (Benjamini and Hochberg 1995) corrected $P \leq 0.005$ among groups in at least one of the groups compared. Gene annotation and GO enrichment analysis for *F. graminearum* was accomplished within the FungiDB database (Basenko et al. 2018; Stajich et al. 2012).

To validate the RNA-Seq gene-expression results, cDNA was synthesized using total RNA with the iScript Reverse Transcription Supermix for reverse transcription-qPCR (Bio-Rad Laboratories Canada Ltd.) as described by the manufacturer. Four microliters of a 50-fold dilution (BC) or a 20-fold dilution (AC) of the cDNA was used in each 20- μl ddPCR reaction along with 10 μl of 2 $\times$ QX200 ddPCR EvaGreen Supermix, 200 nM of each primer, and DNase-free water. Primers were designed to differentiate between genomic DNA and cDNA for most genes (Supplementary Table S2). A no-template control was added for each primer pair. Droplets were generated as per manufacturer instructions (Bio-Rad Laboratories Canada Ltd.) and were transferred to a TwinTec 96-well PCRS45 plate (Eppendorf Canada Ltd.) and were cycled on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories Canada Ltd.), using the

default EvaGreen Supermix amplification protocol at an annealing temperature of 58°C for each primer pair. Optimal annealing temperature was determined by running a temperature gradient for each primer pair ranging from 55 to 61°C . Following amplification, the reaction plate was loaded on the QX200 Droplet Reader for detection. Data were analyzed using the instrument software QuantaSoft version 1.6.6.0320 (Bio-Rad Laboratories Canada Ltd.) and were imported into Microsoft Excel for further analysis and normalization. Expression was normalized with two reference genes, *F. graminearum* *EF1 α* and *β TUB* (Supplementary Table S2).

Differentially regulated gene cluster mining.

C. rosea IK726 transcript identifiers were consecutively numbered according to their relative locations on its genomic sequence (Karlsson et al. 2015). Since secondary metabolite biosynthesis-related genes are typically organized into gene clusters, we took advantage of the assigned transcript identifiers to manually screen the relative expression of transcripts surrounding upregulated secondary metabolite anchor genes—membrane transporter proteins, polyketide, or non-ribosomal peptide synthases. If three or more consecutive transcripts surrounding a putative secondary metabolite gene were up-regulated, their sequences were retrieved and were blasted against the IK726 genome and *C. rosea* CBS125111 to confirm their relative position. Putative cluster products were searched against the FungiSMASH online secondary metabolite prediction tool for verification and prediction (Medema et al. 2011). Up- or downregulated *F. graminearum* gene cluster analysis was based on predicted *F. graminearum* gene clusters (Sieber et al. 2014).

Statistical analysis.

All experiments were replicated at least three times, and one-way analysis of variance and multiple mean separation modules of the GraphPad prism (version 7.0, Graphpad Software) were used to calculate statistical differences and compare the means, respectively.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Ensembl *F. graminearum* database: ftp://ftp.ensemblgenomes.org/pub/fungi/release-36/fasta/fusarium_graminearum
FungiDB database: <https://fungidb.org/fungidb>
IDT primer quest software:
<https://www.idtdna.com/Primerquest/Home/Index>
JGI MycoCosm database:
<https://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Cloro1>
Trimmomatic software v0.36.4:
<http://www.usadellab.org/cms/index.php?page=trimmomatic>

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