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ORIGINAL ARTICLE

Trichoderma atroviride suppresses *Fusarium graminearum* by altering primary and secondary metabolite biosynthesis profiling

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Abstract

The use of *Trichoderma* spp. offers an ecologically friendly tool for the struggle with mycotoxigenic *Fusarium* spp. Here, the alterations in transcriptome level were investigated in 6-day-old *Fusarium graminearum* cultures treated with *Trichoderma atroviride* and nontreated with *T. atroviride* (FGc), using whole transcriptome sequencing to better understand the associated biological processes. Transcriptome analysis indicated a total of 55 up-regulated genes and 728 down-regulated genes with $p_{adj} < 0.05$. Enrichment analysis revealed that the up-regulated genes were related to fatty acid biosynthesis, AMP-dependent biosynthesis, amino acid recognition/activation processes and secondary metabolite production, whereas down-regulated genes were involved in amino acid synthesis, oxidation–reduction processes, metal ion-binding and metabolic/catalytic activities. Among the down-regulated genes, the expression of pigmentation-related genes such as *aurO*, *gip1* and *aurR2* was remarkable. Similarly, the expression levels of key enzyme-coding genes involved in deoxynivalenol mycotoxin production were significantly decreased in the range of −1.77 and −2.94. For up-regulated genes, nonribosomal peptide synthetase and polyketide synthase genes were notably distinguished from the remaining down-regulated genes as these genes can be involved in biosynthesis of common secondary metabolites. The results clearly emphasize that *T. atroviride* repressed the biosynthesis of primary metabolites in *F. graminearum* while simultaneously up-regulating the expression of genes involved in the synthesis of secondary metabolites. This is the first report showing how *T. atroviride* leads to transcriptome alterations and the findings suggest that *T. atroviride* could serve as an effective fungus by employing a wide variety of strategies against phytopathogenic fungi.

KEYWORDS
disease management, *Fusarium graminearum*, Fusarium head blight, RNA-Seq analysis, *Trichoderma atroviride*

1 | INTRODUCTION

Fusarium graminearum, teleomorph *Gibberella zeae*, is the predominant causal agent of Fusarium head blight (FHB) disease of small grain cereals in many agro-ecological regions. This cosmopolitan phytopathogenic fungus has led to epidemics that have cost billions of dollars due to contamination with mycotoxins such as deoxynivalenol and zearalenone as well as reductions in crop quality and quantity (Goswami & Kistler, 2004; Leplat et al., 2013; Lori et al., 2009; Matny, 2015; Miedaner et al., 2008). Its high level of phenotypic

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and genomic plasticity has led to investigations related to *F. graminearum* becoming an important area of research in phytopathology. Moreover, the completed *F. graminearum* genome project has accelerated studies linked to FHB management (Cuomo et al., 2007; Pasquali & Migheli, 2014; Waalwijk et al., 2017; Wang et al., 2011).

F. graminearum is a soilborne phytopathogenic fungus that can persist in the soil for a long time, with a wide distribution pattern worldwide. The predominant strategy for managing *F. graminearum* involves the use of synthetic chemical pesticides. Even if common fungicides provide efficient management of FHB in a short time, there are several disadvantages, such as the development of fungicide resistance and deleterious environmental effects (Alabouvette et al., 2009; Chung et al., 2008; Özsoy et al., 2020; Zheng et al., 2015). In this context, effective and ecologically friendly management strategies, for example the use of plant-derived secondary metabolites such as terpenes, phenylpropanoids, polyketides and alkaloids, are required.

Microbial biological control agents (BCAs) have been frequently used in the fight against *Fusarium* diseases in recent years (Franco et al., 2011; Nourozian et al., 2006). Several fungal species such as *Gliocladium roseum* and *G. catenulatum* may be useful in the in vitro and in vivo control of *F. graminearum* diseases (Er, 2020; Legrand et al., 2018). *Trichoderma* spp. are notable among filamentous fungi for their ability to adapt to a wide variety of ecological conditions and lifestyles, as well as their benefits to plants (Zeilinger et al., 2016). *Trichoderma* species are also important mycoparasitic fungi and compete with other microorganisms for nutrients. *Trichoderma* species including *T. afroharzianum*, *T. atroviride*, *T. harzianum*, *T. virens* and *T. viride* have been used to combat *Fusarium* species. However, the majority of these studies related to interactions between *Trichoderma* spp. and *Fusarium* spp. are limited to two major fungi: *T. harzianum* and *F. oxysporum* (Amin et al., 2010; Mohamed & Haggag, 2006; Rawat et al., 2013; Strauch et al., 2011). Antagonistic effects of *Trichoderma* spp. on *F. graminearum* are only presented in a limited number of investigations. Among these, *T. atroviride* could be evaluated as a potential antagonistic agent against *F. graminearum*, as evidenced by reduced fungal biomass, decreased macroconidium quantity, induced oxidative stress and apoptosis-like-process (Błaszczyk et al., 2017; Cabrera et al., 2020; Matarese et al., 2012; Yörük et al., 2022). However, the response of *F. graminearum* treated with *T. atroviride* at the transcriptomic and/or metabolomic level is not available in the literature. Here, we aimed to reveal *T. atroviride*-induced transcriptomic alterations in *F. graminearum* and to elucidate the biological processes underlying these changes.

2 | MATERIALS AND METHODS

2.1 | In vitro fungal growth

F. graminearum PH-1 and *T. atroviride* TR8 strains were provided by Dr Tapani Yli-Mattila (Finland) and Dr Evrim Özkale (Turkey), respectively; the former was used as the phytopathogen and the latter as the BCA. Fungal cultures were conducted on potato dextrose agar (PDA) at 26 ± 2°C for 6 days. Plugs of mycelium (0.25 cm^2) of *F. graminearum* PH-1 and *T. atroviride* TR8 were transferred onto the middle of the PDA plates. The control set (FGc) included only *F. graminearum* PH-1 grown on PDA, while the experiment set (FGvsTA) consisted of two Petri dishes including both *F. graminearum* PH-1 and *T. atroviride* TR8 cultures. Experimental cultures were conducted as sandwich cultures, in which the Petri dishes (without lids) belonging to two different species were brought together and sealed by Parafilm to form a single plate. Linear growth rates (LGR) of FGc and FGvsTA were measured as mm/day at the end of the seventh day of incubation (Yörük et al., 2022).

2.2 | Total RNA isolation, cDNA-second-strand cDNA synthesis, library preparation and next-generation sequencing

Total RNA was isolated from 7-day-old cultures using a RNeasy Plant Mini Kit (QIAGEN). Fresh mycelial material (50 mg) was first ground with liquid nitrogen using a sterile pestle and mortar. After the homogenization process, total RNA was obtained following the manufacturer's recommendations. Genomic DNA was eliminated by DNase I (QIAGEN) treatment. The quantity and quality of total RNA were checked by Qubit RNA HS Assay Kit (Thermo) and agarose gel (0.8%). RNA integrity was evaluated by Bioptic Qsep100 (GC Biotech). Total RNA samples with sufficient quality were used to prepare cDNA libraries, in equal amounts per sample. Three libraries (one for the control set and two replicates for the experimental culture) were constructed for transcriptome sequencing.

The total RNA was used for first-strand cDNA synthesis with the poly(A) primer using a commercial first-strand cDNA synthesis kit (NEB) including M-MuLV enzyme according to the manufacturer's instructions. After quality check on agarose gel and Qubit fluorometer (Invitrogen-Thermo), the first-strand cDNA was transformed into double-strand (ds) cDNA by using RNase H and DNA polymerase I. Subsequently, the cDNA library fragments of desirable lengths were purified with the AMPure XP beads (Beckman Coulter). The cDNA libraries were then prepared for Illumina sequencing pair-end libraries with insert size of 300 bp using the Illumina DNA Preparation Kit (Illumina Inc.). Thereafter, they were checked and normalized to 10 nM by using Qubit 4.0 and agarose gel (0.8%) electrophoresis. Sequencing was performed on the MiSeq platform (Illumina), conducted at Advanced Technology Application and Research Centre of Sivas Cumhuriyet University (CUTAM, Turkey). The raw data were stored in the NCBI Sequence Read Archive (SRA) under accession number PRJNA970182.

2.3 | Transcriptome data analysis

All sequenced libraries were evaluated in terms of their quality and quantity by using Linux-based tools. The supercomputers with Linux system from TRUBA (Turkish Science e-Infrastructure, TUBITAK-Turkey) sources were used in next-generation sequencing analysis. Sequencing libraries obtained were first subjected to FastQC (and MultiQC) assays in order to check the quality of the reads (http://

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www.bioinformatics.babraham.ac.uk/projects/fastqc/). The trimming process of raw reads was carried out using fastp preprocessor (Chen et al., 2018). After sequencing data were trimmed, the sequenced libraries were used in reference mapping by HiSat2 (Kim et al., 2015). The reads were then mapped to coding regions and counted by using HTseq, Kallisto and StringTie software (Anders et al., 2015; Bray et al., 2016; Pertea et al., 2015). The count tables were transferred to further assays.

2.4 | R-based differentially expressed gene analysis

The count tables belonging to next-generation sequencing (NGS) reads were used in data mining processes by R software v. 4.2.2. DESeq2, apeglm, pheatmap, tidyverse, ggrepel and ashr packages were installed by CRAN or Bioconducter. Count files from previous analyses were normalized using the DESeq2 package. Common commands were used in order to obtain the volcano plot, heatmaps and differentially expressed gene (DEG) table. The genes with an adjusted *p*value (p_{adj}) ≤ 0.05 and a $\log_2$ fold change |1| were accepted/considered as differentially expressed genes. Genes with significantly altered expressions were transferred to functional analysis.

2.5 | Gene ontology and KEGG orthology analysis

Unigenes were analysed with BLASTX algorithm using NCBI, Swiss-Prot (http://web.expasy.org/docs/swissprot_guideline.html), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Cluster of Orthologus Groups (COG) (http://www.ncbi.nlm.nih.gov/COG/) databases. Protein functions were annotated and coding sequence (CDS) regions were confirmed by ETSScan. Unigenes were also subjected to Blast2GO assays. The functional annotations and classifications were carried out using Gene Ontology (GO) (http://geneontology.org/) and WEGO software (https://wego.genomics.cn/). GO trees were constructed from GO Biological process pathway databases with the parameters of false discovery rate (FDR) cut-off value as 0.05 and aspect ratio as 2.0. Blastall was used in obtaining the functional annotations of unigenes by COG and KEGG databases. Metabolic pathway annotations were obtained by KEGG (http://www.genome.jp/kegg/pathway.html). Enrichment analyses were also carried out by GoShiny online tool (http://bioinformatics.sdstate.edu/go).

3 | RESULTS

3.1 | In vitro fungal growth, total RNA isolation, cDNA and second-strand cDNA synthesis

Linear growth rate (LGR) of FGvsTA was compared with FGc. The decreased in vitro growth capacity was obtained by the end of the seventh day of incubation. LGR values of FGc and FGvsTA sets were 12.86 ± 0.01 and 7.71 ± 1.82 mm/day, respectively. A significant decrease in LGR was detected in the FGvsTA set (***$p < 0.01$). Total RNA was isolated from control and experiment sets at a quantity

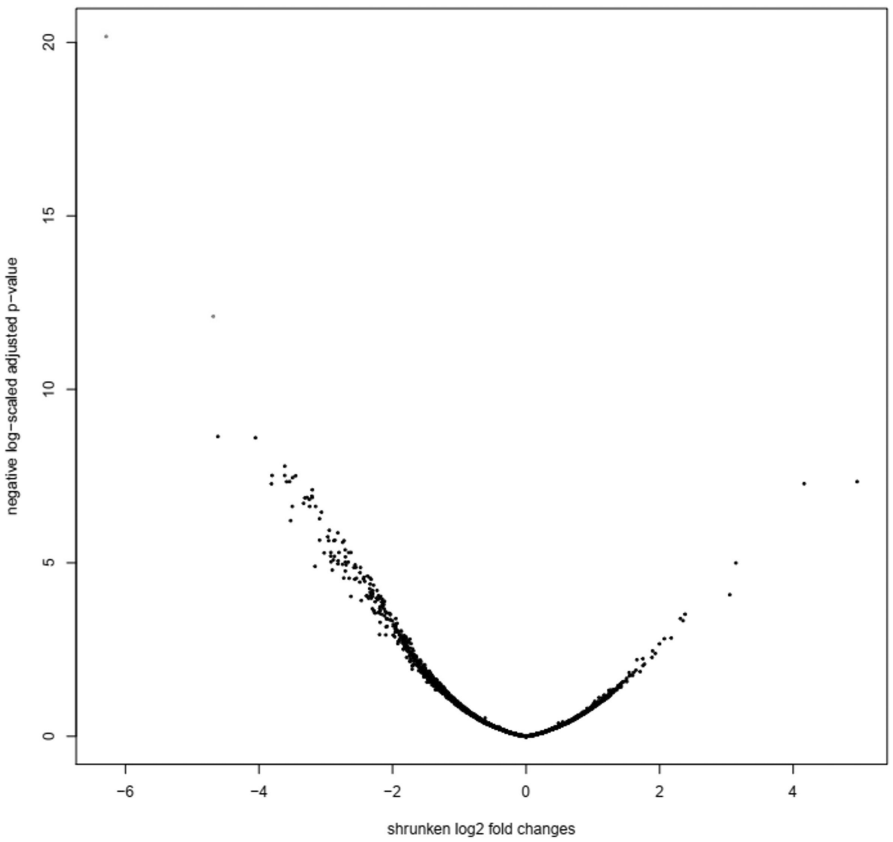

FIGURE 1 Volcano plot generated based on $\log_2$ fold change in *Fusarium graminearum* cultures treated with *Trichoderma atroviride*.

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of 0.5–2 μg/μL and absorbance ratio values of 1.9–2.0 $A_{260/280}$. The quality and the quantity of cDNA and ds cDNA molecules were in the ranges as reported by Nagalakshmi et al. (2010).

3.2 | Transcriptome-wide analysis in *F. graminearum*

Whole transcriptome data for *F. graminearum* PH-1 treated or non-treated with *T. atroviride* were generated to reveal the transcriptomic alterations in the phytopathogenic fungus under the biotic stress factor. Linux-based data were completed by obtaining the count table including Gene-ID numbers, $\log_2$ fold changes with *SE* values, *p* values and p_{adj} values as a single .tsv file (File S1). $p_{adj} \leq 0.05$ was accepted as reference value for identifying the statistically significant differences in genes with differential expression. Minimum p_{adj} values were recorded as 6.8e−21 (FGSG_04745) for down-regulated genes and 4.6e−08 for up-regulated genes (FGSG_11455). In total, 55 up-regulated genes with $p_{adj} \leq 0.05$ were detected, whereas 728 genes exhibited a significant decrease between FGc and FGvsTA sets (File S1). A volcano plot indicated that the majority of the transcripts were not significantly altered (Figure 1). Table 1 highlights the top 10 up-regulated and down-regulated genes with the highest $\log_2$ fold change (FC) values. Up-regulated genes with dramatic fold changes are found in a wide range of different pathways such as actinorhodin (polyketide antibiotic) biosynthetic pathway (FGSG_11455), shikimate pathway (FGSG_11457) and citric acid cycle (FGSG_10444). Table 1 also shows similar striking findings for up-regulated genes involved in antifungal protein synthesis (FGSG_04745), oxidative metabolism of hydrocarbons (FGSG_00043) and sugar transporters (FGSG_05882). DEG analysis revealed that *T. atroviride* led to significant alterations in expression of genes involved in the secondary metabolite biosynthesis in *F. graminearum*. The overall major transcriptomic changes between FGc and FGvsTA and the dramatically up-regulated and/or down-regulated genes are shown in Figures 2 and 3, respectively. Two categorically different GO trees were obtained by Gene Ontology analysis (Figure 4). The GO tree belonging to up-regulated genes included genes predominantly linked to secondary metabolite production processes and fatty acid synthesis (Figure 4a), while the GO tree of down-regulated genes harboured the genes involved in primary metabolite biosynthesis, oxidation–reduction processes and metabolic/catalytic activities (Figure 4b). In this way, the GO trees facilitated the differentiation of different biological processes that involve genes with different critical levels

TABLE 1 Top 20 altered genes in *Fusarium graminearum* treated with *Trichoderma atroviride*.

Gene ID	Log_2FC	p_{adj}	Annotation
Top 10 up-regulated genes			
FGSG_11455	4.96	4.6e−08	Dimeric α-β barrel (IPR011008)
FGSG_10443	4.16	5.2e−08	Hypothetical protein
FGSG_10442	3.14	1.0e−05	Hypothetical protein
FGSG_11457	3.05	8.3e−05	Mmf1/domain is similar in structure to chorismate mutase (IPR006175)
FGSG_10444	2.38	3.0e−04	L-lactate/malate dehydrogenase (PIRSF000102)
FGSG_05048	2.35	4.6e−04	Dihydrodipicolinate synthase (PIRSF001365)
FGSG_13521	2.31	4.0e−04	Hypothetical protein
FGSG_10445	2.17	1.5e−03	Phosphoenolpyruvate synthase (IPR006319)
FGSG_10922	2.07	1.5e−03	Hypothetical protein (with expansin domains [IPR007117, IPR007112])
FGSG_12977	1.94	4.1e−03	Nucleoside triphosphate hydrolase (IPR027417)
Top 10 down-regulated genes			
FGSG_04745	−6.29	6.8e−21	Antifungal protein domain superfamily (IPR023112)
FGSG_09066	−4.68	7.9e−13	Hypothetical protein (with signal peptide)
FGSG_00043	−4.05	2.5e−09	FAD/NAD(P)-binding domain superfamily (IPR036188)
FGSG_07988	−3.58	4.6e−08	Hypothetical protein (with signal peptide)
FGSG_13979	−3.81	5.3e−08	Acyl-CoA *N*-acyltransferase (IPR016181)
FGSG_07896	−3.61	1.6e−08	Chloramphenicol acetyltransferase-like domain superfamily (IPR023213)
FGSG_04527	−3.61	3.0e−08	Carboxypeptidase S1 (IPR001563)
FGSG_08083	−3.45	3.1e−08	Pyridoxal phosphate-dependent decarboxylase (IPR002129)
FGSG_05882	−3.54	4.6e−08	Major facilitator, sugar transporter-like (IPR005828)
FGSG_00053	−3.50	3.5e−08	Aryl-alcohol dehydrogenase (IPR023210)

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of expression. Enrichment assays revealed that up-regulated genes were primarily related to secondary metabolite production steps, whereas down-regulated genes were related to main metabolite synthesis and biological processes with great impact such as amino acid transport, ion transport and oxidation–reduction processes (Figure 5). Table 2 shows the specific genes with significantly altered expression according to enrichment analysis. The enrichment analysis equipped with InterPro database and/or AmiGO was collected as a single.xlsx file consisting of the list of up-regulated and down-regulated genes (File S2).

3.3 | Alterations in expression of genes related to prevalent secondary metabolite biosynthesis

The expression levels of genes encoding key enzymes that regulate biosynthesis of common secondary metabolites highlight the presence of significant changes in *F. graminearum* (Table 3). Moreover, the results of DEG analysis were also considered for the genes related to DON synthesis, aurofusarin synthesis, zearalenone synthesis, apoptosis and autophagy (Figure 6). Figure 6a and File S1 show that majority of the genes involved in apoptosis-like processes did not alter as a result of *T. atroviride* treatment. Similarly, autophagy-related genes did not significantly alter in the FGvsTA set (Figure 6b). However, pigmentation-related aurofusarin biosynthesis genes were significantly down-regulated in response to *T. atroviride* (Figure 6c). Similarly, the genes located in the core synthetic cluster of DON production were significantly decreased (Figure 6d). There were no co-alterations in zearalenone biosynthesis-related genes (Figure 6e). While butanolide production-associated *CYP67* showed down-regulation, up-regulation was observed in some other related key genes of secondary metabolite production (Figure 6f).

4 | DISCUSSION

Today, the whole genome of any kind of organism including large/giant genomes such as *Homo sapiens* could be sequenced in a single day. NGS strategies have evolved from the first-generation whole genome sequencing strategies to third- or fourth-generation sequencing strategies such as Nanopore Sequencing or VASA-Seq transcriptome analysis within the last two decades (Behjati & Tarpey, 2013; Branton et al., 2008; Salmen et al., 2022). RNA-Seq technology uses large-scale parallel sequencing of short cDNA molecules converted from RNA and provides transcriptome reads with high resolution within a short time in comparison with the classical Sanger dideoxy termination method and microarray-based sequencing technologies. Qualitative and quantitative analyses including exon-intron boundaries, haplotyping and transcript levels can be easily carried out by mapping reads to reference genome files (Nagalakshmi et al., 2010). Here, cDNA-based transcriptome analysis in *F. graminearum* treated with *T. atroviride* was carried out in order to discover which strategies the phytopathogen might use in response to a biotic stress factor.

Many of the studies in which *F. graminearum* was the subject of research have been focused on chemotyping, genetic diversity and determination of common fungicide resistance level (Miedaner et al., 2008; Pasquali & Migheli, 2014; Przemieniecki et al., 2014; Wang et al., 2011; Yang et al., 2018; Yörük et al., 2018; Yörük & Yli-Mattila, 2019). Studies on *F. graminearum* at the transcript level have been restricted to alterations of expression of the genes located on the *tri5* gene cluster in response to abiotic stress factors (Boutigny et al., 2009; Gazdağlı et al., 2018; Jiao et al., 2008; Kulik et al., 2014; Marín et al., 2010; Merhej et al., 2010; Oufensou et al., 2020; Pinson-Gadais et al., 2008; Yörük, 2018). There are a few investigations related to transcriptome-wide analysis in *F. graminearum*. Affymetrix

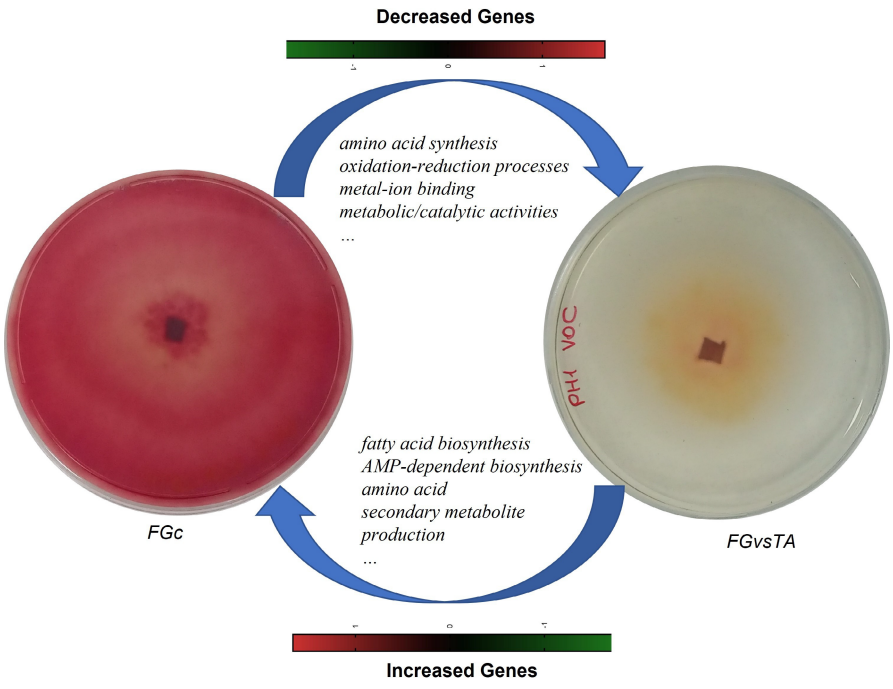

FIGURE 2 Major transcriptomic changes related to primary or secondary biological processes in *Fusarium graminearum* cultures treated with *Trichoderma atroviride* (FGvsTA) compared with untreated *Fusarium graminearum* (FGc). [Colour figure can be viewed at wileyonlinelibrary.com]

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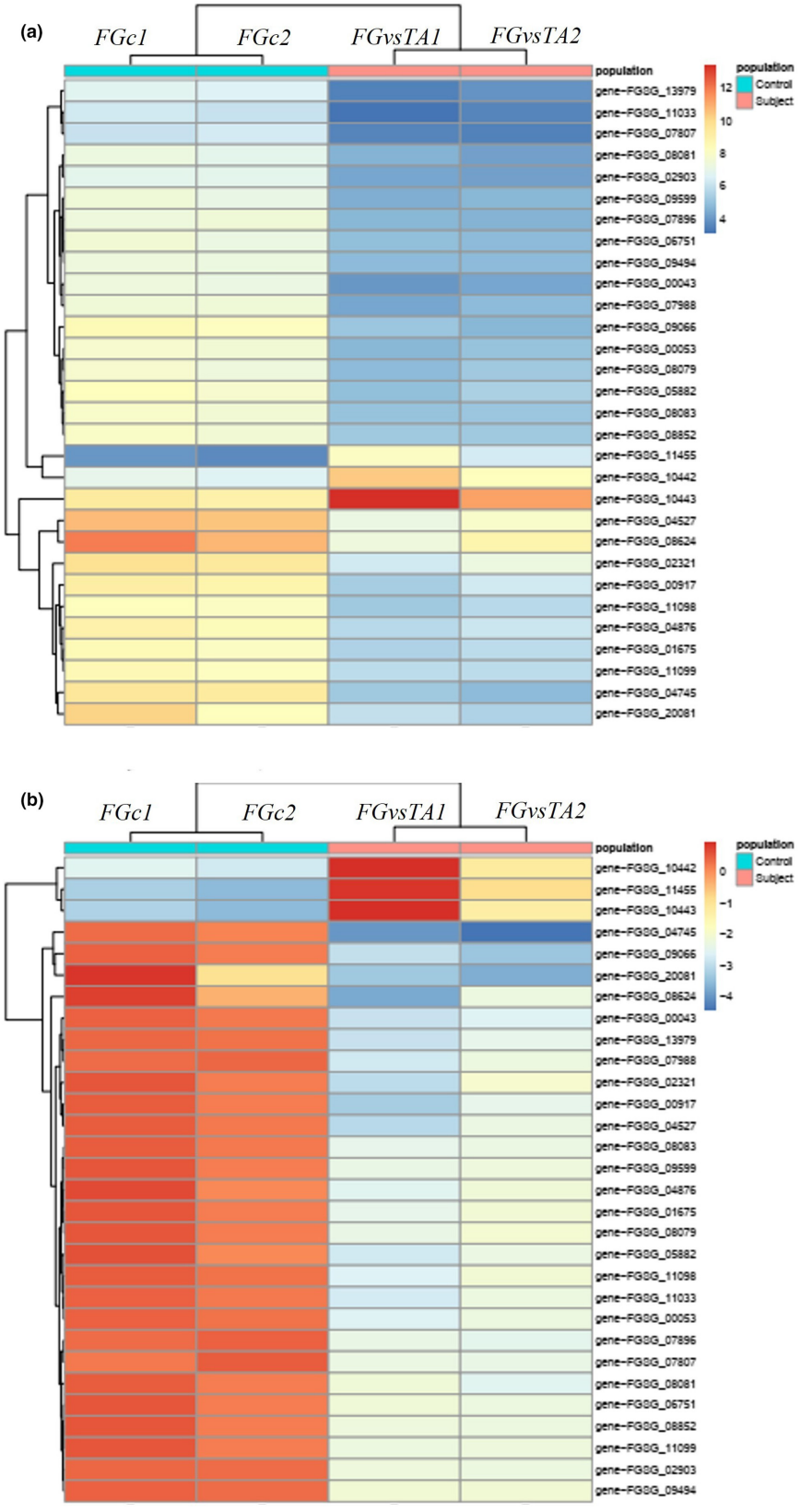

FIGURE 3 Dramatically up-regulated (a) and down-regulated (b) genes. FGc1, FGc2: untreated *Fusarium graminearum* control sets 1 and 2. FGvsTA1, FGvsTA2: *Fusarium graminearum* cultures treated with *Trichoderma atroviride*, sets 1 and 2. [Colour figure can be viewed at wileyonlinelibrary.com]

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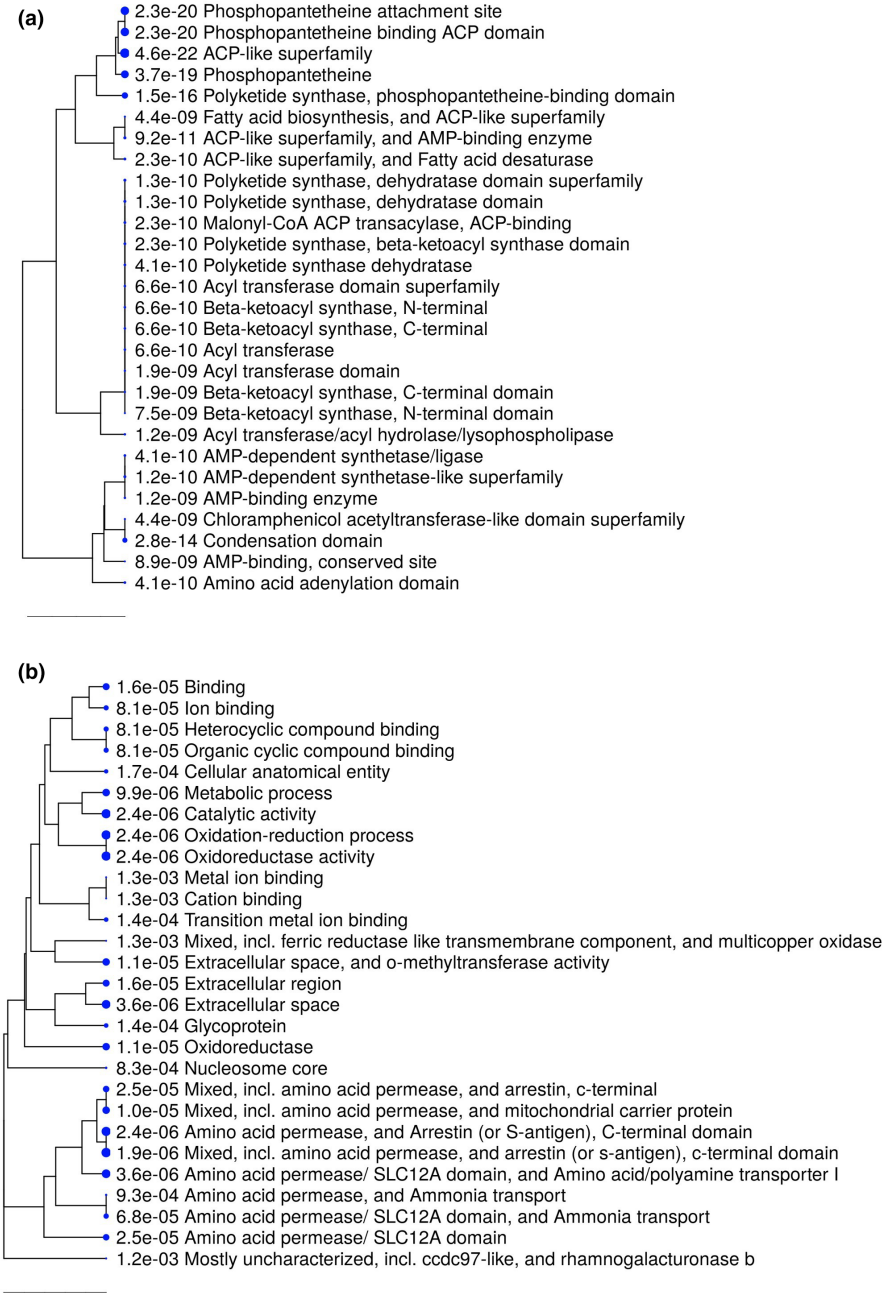

FIGURE 4 Gene Ontology (GO) trees related to up-regulated (a) and down-regulated (b) genes in *Fusarium graminearum* cultures treated with *Trichoderma atroviride*. [Colour figure can be viewed at wileyonlinelibrary.com]

chips and DeepSAGE strategies were developed and conducted as suitable tools for both in vitro and in planta studies in *F. graminearum* (Güldener et al., 2006; Liu et al., 2010). However, these approaches were not followed after high-throughput RNA-Seq-based strategies became more common. Zhao et al. (2013) studied gene annotations in *F. graminearum* by RNA-Seq strategy and reported that alternative splicing strategy processes are regulated by development phases. A holistic approach equipped with RNA-Seq was used in order to reveal the alterations in individual genes or genes located on gene clusters related to secondary metabolite biosynthesis (Sieber et al., 2014). This study revealed that a total of 87 different gene clusters involved in the biosynthesis of known or unknown secondary metabolites showed notable and strong alterations in gene expression specific to enzymatic activity (File S1). Zapparata et al. (2021) investigated the transcriptomic changes in *Trichoderma gamsii* and *F. graminearum* in dual confrontation cultures at the sensing phase of growth. They reported that *F. graminearum* up-regulates the expression of genes related to transmembrane transporters and killer toxins. In total, 653 up-regulated and 75 down-regulated genes with significantly altered expression ($p_{adj} \leq 0.05$ and $\log_2$FC for $>|1|$) were recorded in *F. graminearum* treated with *T. gamsii*. Interestingly, they reported both down-regulation and up-regulation in genes coding for metabolites or ion transporter enzymes, with dramatic up-regulation for MFS general substrate transporter (FGRA07_11673), sugar transporter (FGRA07_06312) and auxin efflux carrier (FGRA07_02452), and notable down-regulation for sugar transporter (FGRA07_11651) and sulphur transport protein (FGRA07_10600) in *F. graminearum* treated with *T. gamsii*.

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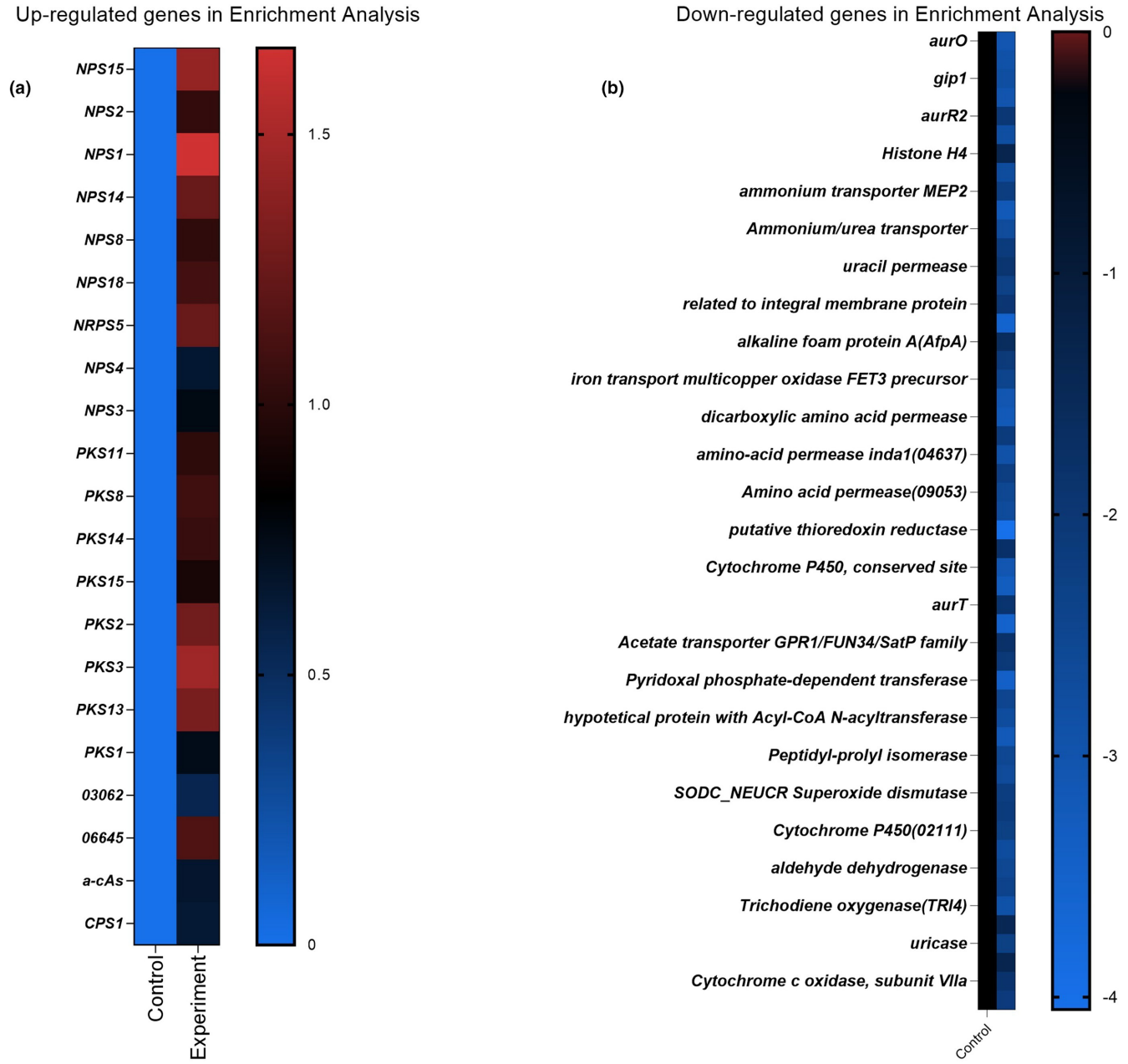

FIGURE 5 Specific transcripts related to characteristic pathways obtained from enrichment analysis. [Colour figure can be viewed at wileyonlinelibrary.com]

Dual culture tests including *F. graminearum* against *T. gamsii* concluded at the sensing phase of fungi (Zapparata et al., 2021). Unlike the mycoparasitism strategy (dual confrontation cultures, "before contact") used by Zapparata et al. (2021), sandwich cultures, which have been preferred in previous studies (Ramangouda et al., 2023; Yörük et al., 2022), were used in this study. These cultures can suppress in vitro growth of *F. graminearum* by up to 40%–50% for 7 days. Zapparata et al. (2021) used *T. gamsii* as BCA, which could be accepted as a strong BCA against *Fusarium* spp. (Baroncelli et al., 2016; Matarese et al., 2012). Here, *T. atroviride* was selected because it has recently been used as a BCA against *Fusarium* spp. (Cabrera et al., 2020; Coninck et al., 2020; Yörük et al., 2022). To our knowledge, this is the first report to show the transcriptome-wide alterations in *F. graminearum* treated with *T. atroviride* under sandwich cultures that could induce the volatile organic compound-based antagonistic effects of *Trichoderma* spp. Here, the number of down-regulated genes in *F. graminearum* was found to be higher than that of a recent report by Zapparata et al. (2021). This may have resulted from the BCA species used or may be due to the mycoparasitism strategy chosen. However, similar transcript-changing profiles were obtained in terms of down-regulation of genes coding for ion and/or metabolite transporter enzymes.

The data obtained from this study contribute novel information to the field by providing a unique perspective on transcript

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TABLE 2 Specific genes with statistically significant alterations obtained from enrichment analysis in *Fusarium graminearum* PH-1 treated with *Trichoderma atroviride*.

Gene-ID	Gene	Log_2FC
Up-regulated genes		
FGSG_02394	NPS15 (nonribosomal peptide synthetase)	1.42
FGSG_11026	NPS1 (nonribosomal peptide synthetase)	1.66
FGSG_11395	NPS14 (nonribosomal peptide synthetase)	1.24
FGSG_11659	NPS8/GRA1 (nonribosomal peptide synthetase)	1.02
FGSG_13783	NPS18 (nonribosomal peptide synthetase)	1.09
FGSG_13878	FgNRPS5 (nonribosomal peptide synthetase)	1.24
FGSG_01790	PKS11 (polyketide synthase)	1.01
FGSG_03340	PKS8 (polyketide synthase)	1.08
FGSG_03964	PKS14 (polyketide synthase)	1.05
FGSG_04694	PKS2 (polyketide synthase)	1.28
FGSG_09182	PKS3 (polyketide synthase)	1.46
FGSG_12126	ZEA2/PKS13 (reducing polyketide synthase)	1.31
FGSG_06645	FGSG_06645 (hypothetical protein)	1.14
Down-regulated genes		
FGSG_02321	aurO (aurofusarin biosynthesis)	−3.09
FGSG_02327	aurF (aurofusarin biosynthesis)	−2.92
FGSG_02328	gip1 (aurofusarin biosynthesis)	−2.82
FGSG_02329	Hypothetical protein with FAS domain	−3.02
FGSG_02323	aurR2 (aurofusarin biosynthesis)	−2.04
FGSG_02326	aurJ (aurofusarin biosynthesis)	−2.81
FGSG_05491	Histone H4	−1.35
FGSG_00702	Nucleotide-diphospho-sugar transferase	−2.75
FGSG_00620	Ammonium transporter MEP2	−2.22
FGSG_01675	Dicarboxylic amino acid permease	−3.21
FGSG_02094	Ammonium/urea transporter	−2.71
FGSG_03412	Amino acid permease inda1	−2.15
FGSG_05277	Uracil permease	−1.94
FGSG_03028	RNA polymerase I-specific transcription initiation factor RRN9	−2.34
FGSG_06118	Related to integral membrane protein	−1.98
FGSG_07988	phiA (cell wall protein)	−3.58
FGSG_08122	afpA (alkaline foam protein A)	−1.63
FGSG_02320	aurR1 (aurofusarin biosynthesis)	−2.12
FGSG_05159	Iron transport multicopper oxidase FET3 precursor	−2.43
FGSG_08079	Benzoate 4-monooxygenase	−3.09
FGSG_03412	Amino acid permease inda1 (03412)	−2.15
FGSG_04637	Amino acid permease inda1 (04637)	−2.90
FGSG_05574	gap1 (general amino acid permease)	−2.26
FGSG_09053	Amino acid permease (09053)	−2.56
FGSG_09297	Amino acid permease/SLC12A domain (09297)	−2.71
FGSG_00043	Putative thioredoxin reductase	−4.05
FGSG_08077	NADPH dehydrogenase YqjM-like	−1.80
FGSG_08079	Cytochrome P450, conserved site	−3.09
FGSG_08081	Isopenicillin N synthase-like, Fe^{2+} 2OG dioxygenase domain (2og-fe)	−3.33
FGSG_02322	aurT (aurofusarin biosynthesis)	−1.92
FGSG_05882	MFS transporter superfamily	−3.54
FGSG_09374	Acetate transporter GPR1/FUN34/SatP family	−1.84
FGSG_03319	Cell division cycle ATPase	−2.09
FGSG_08083	Pyridoxal phosphate-dependent transferase	−3.45
FGSG_00049	Branched-chain amino acid aminotransferase II	−2.49
FGSG_08082	Hypothetical protein with acyl-CoA *N*-acyltransferase	−2.75
FGSG_08852	Putative cryptochrome DASH	−3.20
FGSG_09690	Peptidyl-prolyl isomerase	−2.54
FGSG_05160	Iron permease FTR1	−2.66
FGSG_08721	SODC_NEUCR superoxide dismutase	−2.23
FGSG_02523	Mss4/translationally controlled tumour-associated protein TCTP	−2.16
FGSG_02111	Cytochrome P450 (02111)	−2.30
FGSG_02117	Cytochrome P450 (02117)	−2.73
FGSG_02273	Aldehyde dehydrogenase	−2.56
FGSG_03191	Cytochrome P450 (3191)	−2.42
FGSG_03535	tri4 (trichodiene oxygenase)	−2.95
FGSG_03569	gaoB (galactose oxidase precursor)	−1.46
FGSG_04126	Uricase	−2.30
FGSG_05322	Fatty acid synthase subunit β dehydratase	−1.35
FGSG_09879	Cytochrome c oxidase, subunit VIIa	−1.88
FGSG_10677	Copper amine oxidase	−2.13

profiling of genes involved in secondary metabolite biosynthesis. Genes coding for polyketide synthase were found as mostly up-regulated in response to the presence of biotic stress (Figure 5a). Overall, at least 15 nonribosomal peptide synthase and polyketide synthase genes were up-regulated, representing a sign of the *F. graminearum* survival strategy (Table 2). Among these genes, *nrps* and *pks* genes could be associated with more than one

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TABLE 3 Alterations in the genes involved in common secondary metabolite biosynthesis in *Fusarium graminearum* PH-1 treated with *Trichoderma atroviride*.

Metabolite	Gene ID	Gene	Role	Fold change
Butenolide	FGSG_08079	*CYP67*	CYP67-like; cytochrome P450 family 67 and similar cytochrome P450s	−3.09
Malonichrome	FGSG_11026	*NPS1*	Extracellular siderophore, induced in planta	1.66
Ferricrocin	FGSG_05372	*NPS2*	Intracellular siderophore	1.03
Zearalenone	FGSG_02395 FGSG_12055	*PKS13*	Polyketide synthase; powerful xenoestrogen in animals, no effect on virulence	1.08
Fusarielin	FGSG_10464	*PKS9*	Polyketide synthase	1.56
Aurofusarin	FGSG_02320	*aurR1*	Golden yellow/red pigment of mycelium, low toxicity (high concentrations in feed can affect antioxidant levels in eggs)	−2.12
Orcinol	FGSG_03964	*PKS28*	Responsible for production of orsellinic acid/orcinol	1.05
Deoxynivalenol	FGSG_03537	*TRI5*	Protein biosynthesis inhibitor, virulence factor on wheat	−1.78

secondary metabolite production in phytopathogenic fungi (Frandsen et al., 2006; Gaffoor et al., 2005; Gazdağlı et al., 2023; Ma et al., 2013). Even if the majority of these *nrps* and *pks* genes were associated with the precise pathways, up-regulated genes (such as FGSG_11026, FGSG_02394, FGSG_09182 and FGSG_04694; Table 2) in the FGvsTA experimental set should be used in further studies in evaluating the pathogenomics and pathotranscriptomics in *Fusarium*. Essential genes (*tri4*, *tri5*, *tri9* and *tri14*) related to DON production were down-regulated in the FGvsTA set (Figure 5). The genes of *tri4*, a multifunctional oxygenase responsible for catalysis of four steps in DON synthesis and *tri5*, trichodiene synthase, responsible for the first step in DON biosynthesis, could be regarded as marker genes (Yörük & Yli-Mattila, 2019). The majority of previous studies on the potential decrease in DON biosynthesis focused on the identification of changes in the *tri4* and *tri5* genes (Boutigny et al., 2009, 2010; Merhej et al., 2011; Pinson-Gadais et al., 2008; Ponts et al., 2007; Yaguchi et al., 2009; Yörük & Albayrak, 2019; Yörük et al., 2022). In this manner, a significant decrease in *tri4* and *tri5* genes in FGvsTA experiment sets yielded important data in terms of revealing that *T. atroviride* could be used as a potential effective BCA against *F. graminearum* and related species in further studies. In addition to gene expression alterations related to DON biosynthesis, butanolide mycotoxin biosynthesis-related *CYP67* was also found as down-regulated. However, total transcript changes were not detected in the genes involved in zearalenone production except for *PKS13*. Among the down-regulated genes, the expression of genes linked to aurofusarin biosynthesis had notable expression changes (Figure 6). Almost all genes located on the *AUR* gene cluster were down-regulated. These transcriptomic changes were supported by the Petri dish profiling of the FGvsTA set (Figure 2). It is clear that the lifecycle of *F. graminearum* was disrupted by *T. atroviride* because aurofusarin and its precursors are primarily responsible for the pigmentation and antifungal effects of *F. graminearum* (Frandsen et al., 2006; Gaffoor et al., 2005; Gazdağlı et al., 2023). Additionally, killer toxin genes (FGSG_10551, FGSG_00061, FGSG_00060 and FGSG_00062) exhibited down-regulation or no expression in the FGvsTA set in contrast to the previous report including dual culture tests in *F. graminearum* (Zapparata et al., 2021).

As mentioned earlier, the enrichment analysis showed that many of the genes with up-regulation were associated with secondary metabolite biosynthesis processes. However, here, both GO and KEGG analyses revealed that the down-regulated genes (Figure 5b) belonged to distinct biological processes that are crucial for the survival of *F. graminearum*, such as amino acid metabolism (e.g., FGSG_09053 and FGSG_04637), ammonium/urea transport (e.g., FGSG_02094 and FGSG_00620), ion transport (e.g., FGSG_05159.1 and FGSG_08081) and oxidation–reduction processes (e.g., FGSG_00043 and FGSG_03569). The dramatic alterations (especially down-regulation) in these kinds of genes,

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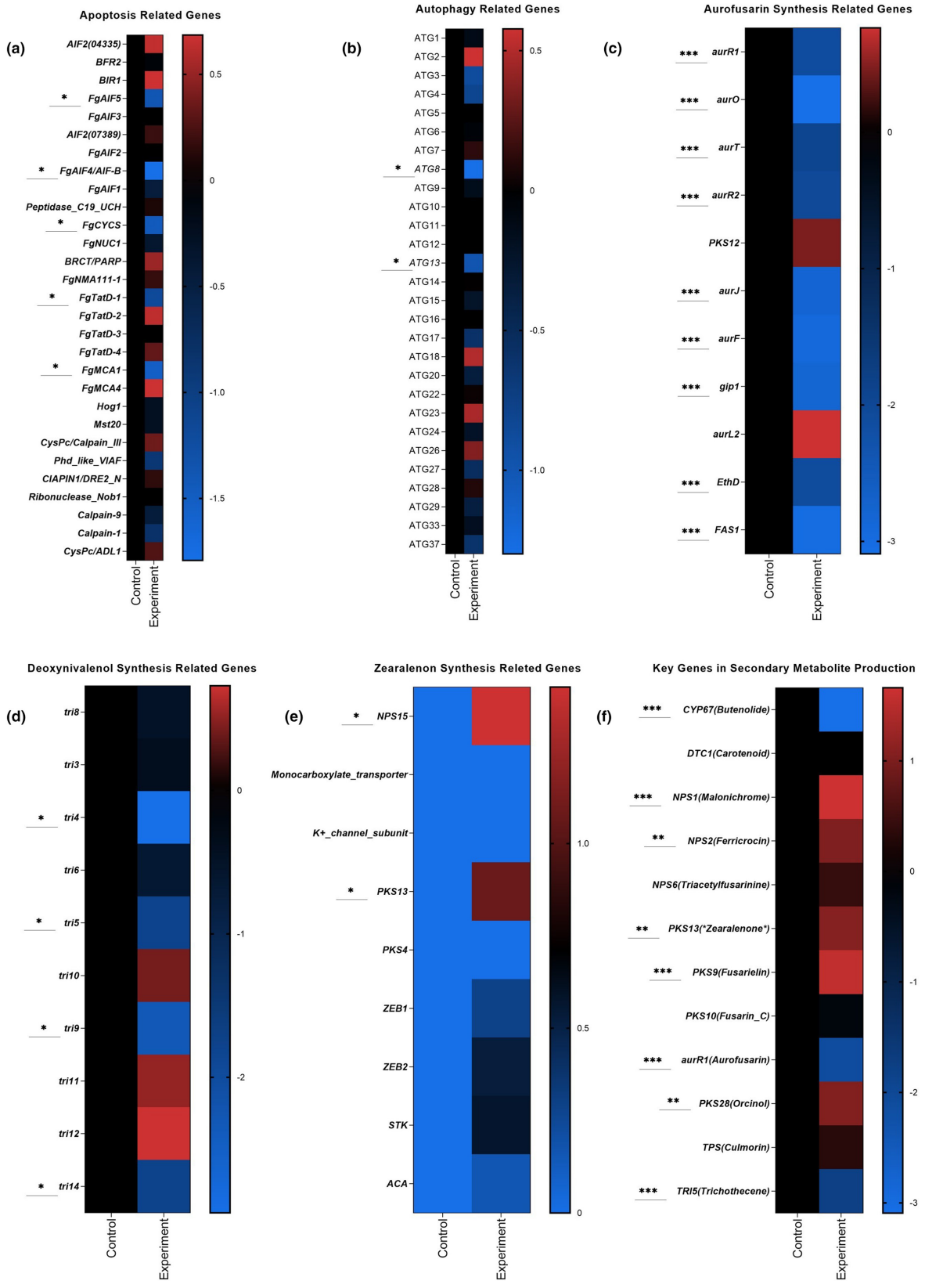

FIGURE 6 Alterations in genes related to apoptosis (a), autophagy (b), aurofusarin synthesis (c), deoxynivalenol synthesis (d), zearalenone synthesis (e) and key enzymes in secondary metabolites (f). Significant difference at $^{*}p_{adj} < 0.05$, $^{**}p_{adj} < 0.01$ and $^{***}p_{adj} < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

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which could be associated with primary metabolism, could be indicative of a poor quality of lifecycle in *F. graminearum*. However, it appears that about 5.3% of down-regulated genes (with p_{adj} value ≤ 0.05 and a $\log_2$ fold change |1|; 728 of a total of 13,694 genes) could not lead *F. graminearum* to programmed cell death (PCD) arising from apoptosis-like process and autophagy (Figure 6). Moreover, no cross-talk was present between apoptosis and autophagy according to the DEG analysis result. Apart from PCD related to apoptosis-like process and autophagy, necroptosis and/or pyroptosis can also initiate PCD in fungi (Gonçalves et al., 2017). However, there is still a lack of information about the genes and pathways involved in these alternate PCD strategies. Necrosis-like cell death regulatory proteins like NWD2 and PNT1 have yet to be annotated and characterized, despite the presence of genomes of *F. graminearum* PH-1 (GCA_000240135.3) and *F. graminearum* ITEM124 (GCA_002352725.1) in databases. We hypothesize that PCD via necroptosis or pyroptosis could be a possible strategy chosen by *F. graminearum* when it is exposed to biotic stress. Further annotation studies are required to fully understand the alternative PCD pathways.

In conclusion, the comprehensive and in-depth investigations here reveal the mechanisms by which *T. atroviride* competes with *F. graminearum*. The majority of the studies on the struggle between *Trichoderma* spp. and *Fusarium* spp. have been conducted on phenotypic and analytical tests (Baroncelli et al., 2016; Benítez et al., 2004; Matarese et al., 2012; Mohamed & Haggag, 2006; Sharma et al., 2013). In this study, transcriptomic alterations were investigated in *F. graminearum* treated with *T. atroviride* in sandwich cultures. It was shown that *T. atroviride* decreases the expression of genes involved in key biological pathways in *F. graminearum*. This was also demonstrated by a disruption in the abundance of transcripts related to the processes of mycotoxin biosynthesis, ion/metabolite transport, amino acid metabolism and oxidation–reduction. Based on the findings of this study, *T. atroviride* has the potential to be an effective BCA that employs a wide variety of strategies to fight against phytopathogenic microorganisms.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data that support this work have been deposited in NCBI BioProject at https://www.ncbi.nlm.nih.gov/bioproject/ with accession number PRJNA970182.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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