Effects of Peptide C_{12}-OOWW-NH_{2} on Transcriptome and Cell Wall of the Postharvest Fungal Pathogen Penicillium digitatum

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In this study, the transcriptional profiling of Penicillium digitatum after C_{12}O_{3}TR treatment was analyzed by RNA-Seq technology. A total of 2562 and 667 genes in P. digitatum were differentially expressed after 2 and 12 h treatment, respectively. These genes were respectively mapped to 91 and 79 KEGG pathways. The expression patterns of differentially expressed genes (DEGs) at 2 and 12 h were similar, mainly were the metabolic processes in cell wall, cell membrane, genetic information and energy. Particularly, the main metabolic process which was affected by C_{12}O_{3}TR stress for 2 and 12 h was cell integrity, including cell wall and cell membrane. The changes of chitin in cell wall was observed by Calcofluor White (CFW) staining assay. The weaker blue fluorescence in the cell wall septa, the decrease of β-1, 3-glucan synthase activity and the increase of chitinase and AKP activity showed that C_{12}O_{3}TR could damage the cell wall integrity. In conclusion, these results suggested that C_{12}O_{3}TR could inhibit the growth of P. digitatum through various mechanisms at transcriptional level, and could influence the cell wall permeability and integrity.

Keywords: Penicillium digitatum, peptide, C_{12}O_{3}TR, RNA-Seq, cell wall

INTRODUCTION

During postharvest storage and marketing process, citrus fruit usually suffers significant economic losses mainly due to the green mold disease which was caused by Penicillium digitatum (Droby et al., 2008; Lu et al., 2018). Conventional chemical fungicides are highly effective against this pathogen, and therefore are commonly used to control the green mold disease on citrus fruit. However, intense application of chemical fungicides has aroused the concerns to the environment, human health, and development of fungicide resistance strains. Therefore, there is an urgent need to replace or reduce the use of chemical fungicides by effective and eco-friendly methods (Palou et al., 2016; Romanazzi et al., 2015).

Antimicrobial peptides (AMPs) are one of the strong candidates. AMPs are gene-encoded, ribosomally synthesized polypeptides, which are cationic or anionic (Maget-Dana and Peypoux, 1994; Mulder et al., 2013). AMPs are widely present in plants, animals, insects and microorganisms with a broad spectrum of activity against viruses, bacteria, fungi, and parasites (Jenssen et al., 2006). They are also important components against invading pathogens in the biological innate immunity system (López-Garcia et al., 2015; Wang et al., 2018a). Because of their efficient control
effects against pathogens, less developmental resistance and low toxicity to host cells, they have been proposed as novel antibiotics in many fields, such as agriculture, animal husbandry and the food industry (Jenssen et al., 2006; Keynanesh et al., 2009; Ciociola et al., 2016). Currently, an increasing number of researchers are trying to use AMPs to control postharvest diseases of fruit and vegetable (Johnson et al., 2015; Puig et al., 2016). There are more and more rationally designed and chemically synthesized peptides which have been proven to be effective against postharvest pathogens, such as BP15, PAF26 and MsrA1 (Osusky et al., 2000; Muñoz et al., 2007; Puig et al., 2016).

Peptide C12-OOWW-NH2 (C12O3TR) which was synthesized by combining peptide O3TR (H-OOWW-NH2) with saturated fatty acids, could effectively inhibit some clinically important bacteria (such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*) and postharvest pathogens (such as *Penicillium expansum*, *Aspergillus niger*, and *Fusarium culmorum*) (Laverty et al., 2010; Thery et al., 2018). Our previous study showed that C12O3TR could significantly suppress the growth of *P. digitatum* with the minimum inhibitory concentration (MIC) of 6.25 μmol/L. But the mechanism of C12O3TR against *P. digitatum* was still unclear. The classic antifungal mechanism mainly focuses on the interaction between AMPs and cell membrane (Abedinazadeh et al., 2015; Omardien et al., 2016). While some researches have proposed other mechanisms which pointed out that some AMPs could interact with intracellular specific targets, such as DNA, RNA and protein or could interfere the synthesis of cell wall to inhibit the growth of pathogens or could induce reactive oxygen species (ROS) production in cell (Petruzzelli et al., 2003; Scocchi et al., 2016; Shah et al., 2016). Recent research has found that C12O3TR could inhibit the growth of fungi by changing the membrane permeabilization (Li et al., 2019), but whether there are any other antifungal mechanisms needs to be further clarified.

RNA-Seq is an innovative technology for the quantification and identification of gene expression. Due to its sensitivity, high resolution and comprehensive features, it has become increasingly popular in various studies which aim to reveal the change of the organism gene expression in different environment (Nookaew et al., 2012; OuYang et al., 2016). RNA-seq has also been used to explore the molecular mechanism of fungal drug-resistance or fungi-host interaction (Liu et al., 2015; Barad et al., 2016; Wang et al., 2016). There are several studies that utilized RNA-Seq technology to investigate the fungal response mechanism to peptide such as MAF-1A (Wang et al., 2017).

The objective of this work was to reveal the molecular antifungal mechanism of C12O3TR against *P. digitatum* by using high-throughput RNA-Seq technique, and to confirm the effect of C12O3TR on cell wall at superficial level.

**MATERIALS AND METHODS**

**Fungal Species**

*Penicillium digitatum* was used in this work and cultured on potato dextrose agar (PDA) plates at 25°C (Wang et al., 2018b; Liu S. et al., 2019; Liu Y. et al., 2019). Fungal conidia suspension was obtained by flooding the 7-days-old culture spores with sterile distilled water, followed by filtering through four layers of sterile gauze, and then adjusting to the suitable concentration.

**Peptide Synthesis**

The peptide (C123H23O)OOWW-NH2 (C12O3TR) was purchased from GenScript Corporation (Nanjing, China) with > 90% purity. The purity was selected based on other relevant studies and cost consideration (Laverty et al., 2015; Thery et al., 2018). Peptide C12O3TR was synthesized by the solid-phase methods using 9-fluorenylmethoxy carbonyl (Fmoc)-type chemistry. C12O3TR was amidated at the C terminus. Stock solutions of peptide was reconstituted at 1 mmol/L by using sterile distilled water (C12O3TR could completely dissolved by using sterile distilled) and then stored at −40°C. C12O3TR was not sensitive to oxidation.

**Preparation of C12O3TR Treatments**

*P. digitatum* conidia (1 × 10⁵ CFU/mL, 100 μL) were inoculated in 20 mL potato dextrose broth (PDB), and incubated at 25°C in thermostatic shaker at 160 rpm for 2 d. The *P. digitatum* mycelia were obtained by centrifuging at 4000 × g for 15 min. After washing with PBS (pH 7.0) 3 times, the mycelia were resuspended in 20 mL PBS (pH 7.0). Subsequently, the peptide C12O3TR was added into the suspensions to the final concentration of 6.25 μmol/L (MIC), and then incubated at 25°C for 0, 2, 4, 6, and 12 h. PBS (pH 7.0) was used as the control. Each treatment was repeated three times. Finally, the mycelia samples which removed PBS were immediately frozen in liquid nitrogen and stored at −80°C until use.

**RNA Extraction and Illumina Sequencing**

The *P. digitatum* mycelia samples after 2 and 12 h of C12O3TR and PBS treatment were used for this experiment. The four treatments were named C2, C12O3TR2, C12, C12O3TR12, respectively. Total RNA preparation, RNA quality detection, cDNA libraries construction and RNA-seq were carried out by using a service from Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Total RNA was extracted by using TRIZol reagent (Invitrogen, United States) according to the manufacturer’s instructions. RNA contamination and degradation were monitored on 1% agarose gels. The NanoPhotometer® spectrophotometer (Implen, CA, United States) was used to check RNA purity. RNA integrity was assessed by using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, United States) (Wang et al., 2017).

One μg RNA per sample was used as input material for the RNA sample preparations. NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, United States) following manufacturer recommendations was used to generate sequencing libraries and index codes were added to attribute sequences to each sample. Then the cDNA libraries were sequenced by using an Illumina HiSeq2000™ platform (Lin et al., 2013; OuYang et al., 2016; Lai et al., 2017). The resulting RNA-seq reads were mapped onto the reference genome of *Penicillium digitatum* Pd1.
(GCA_000315645)\(^1\) (Marcet-Houben et al., 2012). Gene model annotation files and reference genome were directly downloaded from genome website.\(^2\) Index of the reference genome was built by using Hisat2 v2.0.5. Paired-end clean reads were aligned to the reference genome by using Hisat2 v2.0.5.

In order to identify differentially expressed genes (DEGs) between C2 and C\(_{12}\)O\(_3\)TR2, or C12 and C\(_{12}\)O\(_3\)TR12, transcript abundance was estimated by using the method of expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FRKM) (Trapnell et al., 2010). Differential expression analysis of two conditions/groups (three biological replicates per condition) was performed by using the DESeq R package.\(^1?\) The P-values were adjusted by using the Benjamini and Hochberg method. P-value of 0.05 and absolute fold change of 2 were set as the threshold for significantly differential expression (Wang et al., 2010).

Gene Ontology (GO) enrichment analysis of DEGs were implemented by using the clusterProfiler R package. GO terms with corrected P-value (padj) < 0.05 were considered significantly enriched (Young et al., 2010). In order to identify the biological pathways which were active in \(P.\ digitatum\), all DEGs were mapped to the reference canonical pathways contained in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.\(^3\) The statistical enrichment of DEGs in KEGG pathways were tested by using clusterProfiler R package (Mao et al., 2005). The two-tailed Fisher exact test based on the false discovery rate (FDR) cutoff of 0.05 was used as one of the justification conditions.

### Quantitative Real-Time PCR (qRT-PCR) Analysis

Ten DEGs were selected in this study to validate the results of RNA-Seq. The RNA, which used for quantitative reverse transcription PCR (qRT-PCR) analysis, was extracted by using the same method of 2.3 and qualified by Nanodrop 2000 Spectrophotometer (Thermo-Fisher scientific Inc., Wilmington, DE, United States). cDNAs were constructed from 1 \(\mu\)g total RNA by reverse transcription using the PrimeScript\(^\text{TM}\) RT Reagent Kit with gDNA Eraser (TAKARA, Tokyo, Japan). qRT-PCR analysis was performed as described by Zhou Y. et al. (2018), with some modifications. Briefly, 20 \(\mu\)L reaction system of SYBR Green PCR Master Mix (Applied Biosystems, United States) and the Step One Plus Real-time PCR System (Applied Biosystems, United States) were used for the qRT-PCR analysis. Reaction procedures were started at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and then hold at 60°C for 30 s. The primers for the qRT-PCR were synthesized by Sangon Biotech (Shanghai, China) and presented in Supplementary Table S1. The changes in SYBR Green fluorescence in every cycle were monitored, and the threshold cycle (Ct) over the background were calculated for each reaction. The relative expression level of the genes were calculated using the 2 \((-ΔΔCt)\) method (Livak and Schmittgen, 2001).

\(^1\)http://fungi.ensembl.org/Penicillium_digitatum_pdi1_gca_000315645/Info/ Index
\(^2\)https://www.ncbi.nlm.nih.gov/genome/13384
\(^3\)http://www.genome.jp/kegg/

The aim of experiment was mainly qualitatively verified the reliability of the RNA-Seq data by qRT-PCR. The actin gene (PDIP_27720) was utilized as the internal reference to normalize the expression data (OuYang et al., 2016). Each PCR reaction was repeated three times, and there were three parallel sets for each reaction.

### Assays for Alkaline Phosphatase (AKP) Activity

The AKP activity of \(P.\ digitatum\) mycelia was determined by AKP kit (Solarbio Science and Technology Co., Ltd., Beijing, China). The \(P.\ digitatum\) mycelia samples after 0, 2, 4, 6, and 12 h of \(C_{12}\)O\(_3\)TR treatment were used for this experiment. PBS (pH 7.0) was used as the control. Each experiment was repeated three times. One unit of AKP activity was defined as the time (min) to produce 1 \(\mu\)mol phenol per 1 g \(P.\ digitatum\) mycelia sample at 37°C.

### Assays for β-1, 3-Glucan Synthase Activity

β-1, 3-glucan synthase activity was analyzed as described by Moreno-Velásquez et al. (2017), with some modifications. Four hundred mg \(P.\ digitatum\) mycelia samples were homogenized with 0.1 mol/L citrate - 0.2 mol/L disodium phosphate buffer (pH 4.8, 1.5 mL) in liquid nitrogen. The mixture was then centrifuged at 12000 \(\times\) g for 10 min at 4°C, and the supernatant was collected as the enzyme extract liquid. After that, 1 mL enzyme extract liquid and 1 mL 0.1 mol/L citrate - 0.2 mol/L disodium phosphate buffer (pH 4.8) were mixed with fucoidan solution (1 mg of fucoidan dissolved in 1 mL distilled water), and then the solution was incubated at 30°C water bath for 1 h. Then, distilled water (1.5 mL), anthrone ethyl acetate (0.5 mL) and \(H_2SO_4\) (3 mL) were respectively orderly added in 0.5 mL solution which was after 30°C water bath. The whole mixture was heated in boiling water bath for 1 min, and then cooled down to room temperature to measure the absorbance at 630 nm. One unit of β-1, 3-glucan synthase activity was defined as the time (hour) to produce 1 mg glucose per 1 g \(P.\ digitatum\) mycelia sample at 30°C. The glucose content standard curve was calculated based on glucose content (x axis) against absorbance value (y axis) \((y = 1.662x + 0.1545, R^2 = 0.9904)\).

### Assays for Chitinase Activity

Chitinase activity was analyzed as described by Pan et al. (2020), with some modifications. The samples after 0, 2, 4, 6, and 12 h of \(C_{12}\)O\(_3\)TR treatment were used for this experiment. Two hundred mg \(P.\ digitatum\) mycelia samples were homogenized in liquid nitrogen with acetic acid buffer (1 mL), and then centrifuged at 12000 \(\times\) g for 10 min at 4°C. The supernatant was collected as enzyme extract liquid. The enzyme extract liquid (0.4 mL) and acetic acid buffer (0.4 mL) were mixed with colloidal chitin solution, and then the mixture was incubated at 37°C water bath for 2 h. The reaction system was stopped by centrifuging at 4000 \(\times\) g for 10 min. Afterward, 0.4 mL of the supernatant was mixed with saturated borax solution (0.2 mL), and then the mixture was heated in boiling water bath for 7 min. After cooling to room temperature, glacial acetic acid (2 mL) and
1% p-dimethylaminobenzaldehyde (DMAB) (1 mL) were added in the solution which was cooled to room temperature. The absorbance was measured at 585 nm, and the chitinase activity was calculated based on the standard curve. One unit of chitinase activity was defined as the time (hour) to produce 1 µg of N-acetylglucosamine (GlcNAc) per 1 g of P. digitatum mycelia at 37°C. The standard curve of GlcNAc content was established according to GlcNAc content (x axis) against the absorbance value (y axis) \( y = 0.0115x + 0.0359, R^2 = 0.9974 \).

The Effect of C\textsubscript{12}O\textsubscript{3}TR on the Cell Wall Integrity of P. digitatum

The effect of C\textsubscript{12}O\textsubscript{3}TR on P. digitatum cell wall integrity was determined as described by OuYang et al. (2019), with some modifications. P. digitatum conidia (1 \( \times \) 10\textsuperscript{4} CFU/mL, 90 µL) mixed with 5% PDB were cultured at 25°C for 48 h. C\textsubscript{12}O\textsubscript{3}TR with the final concentrations of 6.25 µmol/L (MIC) was added and then incubated for 0, 2, and 12 h. PBS (pH 7.0) was used as control. Each sample was then stained with 50 mg/L Calcefluor White (CFW) for 5 min in dark, and the fluorescence was examined and photographed by the Eclipse TS100 epifluorescence microscope (Nikon Corporation, Japan) with DAPI filter sets. The reproducibility of experiment results was confirmed by three replicates.

Statistical Analysis

All experiments included three parallel sets and were repeated three times. The data were processed with Microsoft Excel 2013, and analyzed by statistical software SPSS 21.0 (SPSS Inc., Chicago, IL, United States). The variance of data was analyzed via one-way analysis of variance (ANOVA) with Duncan’s multiple range tests at \( p < 0.05 \).

RESULTS

RNA Sequencing and Gene Prediction

The profile of transcriptome sequence average data was shown in Table 1. The objective data for sequencing each sample was shown in Supplementary Table S2. Based on RNA-seq, an average of 63.93 ± 4.00 million, 66.06 ± 6.00 million, 58.83 ± 2.72 million and 57.94 ± 2.26 million raw reads were generated from C2, C\textsubscript{12}O\textsubscript{3}TR2, C12, and C\textsubscript{12}O\textsubscript{3}TR12 samples, respectively. After filtering the adaptor sequences, the average clean reads were 62.50 ± 4.62 million, 64.98 ± 5.89 million, 57.76 ± 2.65 million and 56.47 ± 1.86 million for the four treatment groups, respectively. Among them, 94.87 ± 0.08%, 95.06 ± 0.22%, 95.23 ± 0.10% and 95.21 ± 0.16% of the total reads were mapped to the genome of P. digitatum in C2, C\textsubscript{12}O\textsubscript{3}TR2, C12, and C\textsubscript{12}O\textsubscript{3}TR12 samples, respectively. In Supplementary Table S2, the percentages of the 12 sequencing samples total reads mapped to the genome of P. digitatum were all more than 90%. And 94.39 ± 0.10%, 94.60 ± 0.22%, 94.66 ± 0.08% and 94.61 ± 0.14% of the reads were uniquely mapped. In addition, the mapped reads of 12 samples represented the filtered data were all less than 1%, in Supplementary Table S2. In conclusion, none of the sequencing samples were contaminated, and all the experimental samples met the requirements of subsequent experiments.

3.2 Transcriptional Stress Response of P. digitatum to peptide C\textsubscript{12}O\textsubscript{3}TR

The RNA sequencing results in Figure 1 revealed the differences in distribution and density distribution of gene expression in C2, C\textsubscript{12}O\textsubscript{3}TR2, C12 and C\textsubscript{12}O\textsubscript{3}TR12 samples. There were also differences in gene expression distribution among three biological replicates under the same treatment (Figure 1A). The gene expressions of all groups had the same characteristics, which were, most of the genes were low in expression, while a few genes were high in expression (Figure 1B). In conclusion, these phenomena were consistent with the law of gene expression in biology. The volcano plots of the DEGs demonstrated that there were 2562 genes which were differentially expressed in C\textsubscript{12}O\textsubscript{3}TR treatment, including 1313 up-regulated genes and 1249 down-regulated genes (Figure 2A). In addition, a total of 667 DEGs were detected between C12 and C\textsubscript{12}O\textsubscript{3}TR12, in which 361 genes were up-regulated and 306 were down-regulated (Figure 2B). To further analyze the effect of different time on gene expression of P. digitatum mycelia in response to C\textsubscript{12}O\textsubscript{3}TR, DEGs in groups treated with C\textsubscript{12}O\textsubscript{3}TR for 2 and 12 h were compared. The results

### Table 1 | Profile of the transcriptome sequence data.

| Parameter                  | C2          | C\textsubscript{12}O\textsubscript{3}TR2 | C12          | C\textsubscript{12}O\textsubscript{3}TR12 |
|----------------------------|-------------|---------------------------------------|--------------|----------------------------------------|
| Raw reads (million)        | 63.93 ± 4.00| 66.06 ± 6.00                          | 58.83 ± 2.72 | 57.94 ± 2.26                           |
| Clean reads (million)      | 62.50 ± 4.62| 64.98 ± 5.89                          | 57.76 ± 2.65 | 56.47 ± 1.86                           |
| Clean bases (G)            | 9.38 ± 0.69 | 9.75 ± 0.89                           | 8.67 ± 0.40  | 8.47 ± 0.28                            |
| Error rate (%)             | 0.02 ± 0.00 | 0.03 ± 0.00                           | 0.02 ± 0.00  | 0.02 ± 0.00                            |
| Q20 (%)                    | 98.14 ± 0.22| 97.97 ± 0.27                          | 98.33 ± 0.02 | 98.31 ± 0.06                           |
| Q30 (%)                    | 94.63 ± 0.45| 94.33 ± 0.55                          | 95.05 ± 0.04 | 94.96 ± 0.14                           |
| GC content (%)             | 53.78 ± 0.00| 53.62 ± 0.10                          | 53.64 ± 0.07 | 53.58 ± 0.06                           |
| Total mapped reads         | 59291296 (94.87 ± 0.08%) | 61780893 (95.06 ± 0.22%) | 55008480 (95.23 ± 0.10%) | 53768470 (95.21 ± 0.16%) |
| Uniquely mapped reads      | 58985616 (94.39 ± 0.10%) | 61483597 (94.60 ± 0.22%) | 54675270 (94.66 ± 0.08%) | 53428164 (94.61 ± 0.14%) |
| Multiple mapped reads      | 306582 (0.49 ± 0.02%) | 297296 (0.48 ± 0.01%) | 333210.7 (0.57 ± 0.02%) | 340306.3 (0.60 ± 0.05%) |

The samples C2 was control group, and the C\textsubscript{12}O\textsubscript{3}TR2 samples belonged to the group exposed to peptide C\textsubscript{12}O\textsubscript{3}TR at MIC after 2 h. Similarly, C12 was control group, and C\textsubscript{12}O\textsubscript{3}TR12 was the group which exposed to C\textsubscript{12}O\textsubscript{3}TR at MIC after 12 h. Q20 and Q30 are the percentage of quality values greater than or equal to 20 or 30.
FIGURE 1 | The comparison diagram of gene expression level. (A) Gene expression distribution. The x-axis shows the name of samples, and C2-1, C2-2 and C2-3 represent three biological duplicate. The y-axis shows the value of log₂(FPKM+1). (B) FPKM density distribution. The x-axis shows the value of log₂(FPKM+1). The y-axis shows the density of samples.
showed that 303 genes were consistently differentially expressed in the two treatments (Figure 2C).

**Enrichment Analysis of GO**

GO analysis was used to classify the DEGs of *P. digitatum* in response to C$_{12}$O$_{3}$TR. A total of 2562 DEGs were mapped to 572 GO terms in sample treated with C$_{12}$O$_{3}$TR for 2 h. Among them, 308, 74, and 190 GO terms were assigned to biological process, cellular component, and molecular function, respectively. Figure 3A showed the top 30 enriched functional categories of the 2562 DEGs. The part information of the significant enrichment terms were shown in the Supplementary Table S3. As could be seen, the significant enrichment terms in biological process included cellular amide metabolic process, amide biosynthetic process, cellular protein metabolic process, peptide biosynthetic process, peptide metabolic process, translation and protein metabolic process. Significant enrichment terms in cellular component were ribonucleoprotein complex, ribosome, cytoplasm, cytoplasmic part, non-membrane-bounded organelle and intracellular non-membrane-bounded organelle. And the significant enrichment terms in molecular function were mostly structural constituent of ribosome and structural molecule activity.

After 12 h C$_{12}$O$_{3}$TR treatment, 667 DEGs were mapped to 572 GO terms (Figure 3B), in which 201, 46, and 165 GO terms were assigned to biological process, cellular component and molecular function, respectively. The part information
**FIGURE 3** | GO functional classification analysis of DEGs. GO functional classification of DEGs in *P. digitatum* under C12O3TR (MIC) stress after 2 h (A) and 12 h (B) of culturing. X axis means GO term. Y axis represents the value of $-\log_{10}(padj)$. The number on each pillar is the number of DEGs in each GO term. All GO terms are grouped into three ontologies: red is for biological process (BP), green is for cellular component (CC), and blue is for molecular function (MF). The ** symbol located at the figure indicated that the GO term was significantly enriched ($padj < 0.05$).
### TABLE 2 | Mostly enriched KEGG pathway of DEGs in *P. digitatum*.

| Pathway                                             | Input number | Background number | Pathway ID  |
|-----------------------------------------------------|--------------|-------------------|-------------|
| **2 h**                                             |              |                   |             |
| Ribosome                                            | 44           | 82                | pcs003010   |
| Citrate cycle (TCA cycle)                           | 14           | 26                | pcs00020    |
| Pyruvate metabolism                                 | 16           | 32                | pcs00620    |
| Carbon metabolism                                   | 41           | 102               | pcs01200    |
| beta-Alanine metabolism                              | 11           | 21                | pcs00410    |
| Glyoxylate and dicarboxylate metabolism              | 16           | 35                | pcs00630    |
| Nitrogen metabolism                                 | 8            | 15                | pcs00910    |
| Alanine, aspartate and glutamate metabolism         | 13           | 29                | pcs00250    |
| Sphingolipid metabolism                             | 8            | 16                | pcs00600    |
| Glycine, serine and threonine metabolism            | 16           | 38                | pcs00260    |
| Spliceosome                                          | 32           | 85                | pcs03040    |
| Fructose and mannose metabolism                     | 12           | 28                | pcs00510    |
| Pantothenate and CoA biosynthesis                    | 9            | 20                | pcs00770    |
| Ether lipid metabolism                               | 6            | 12                | pcs00565    |
| SNARE interactions in vesicular transport           | 6            | 12                | pcs04130    |
| Glutathione metabolism                              | 11           | 26                | pcs00480    |
| Valine, leucine and isoleucine degradation           | 13           | 32                | pcs00280    |
| 2-Oxocarboxylic acid metabolism                     | 14           | 35                | pcs01210    |
| N-Glycan biosynthesis                               | 11           | 27                | pcs00510    |
| Methane metabolism                                  | 8            | 19                | pcs00680    |
| Phenylalanine metabolism                            | 9            | 22                | pcs00360    |
| Amino sugar and nucleotide sugar metabolism         | 17           | 48                | pcs00520    |
| Pentose phosphate pathway                           | 9            | 24                | pcs00030    |
| Fatty acid biosynthesis                             | 5            | 12                | pcs00061    |
| Thiamine metabolism                                 | 5            | 12                | pcs00730    |
| Pentose and glucuronate interconversions            | 8            | 21                | pcs00040    |
| Biosynthesis of secondary metabolites               | 95           | 301               | pcs01110    |
| Cysteine and methionine metabolism                  | 15           | 43                | pcs00270    |
| Tryptophan metabolism                               | 11           | 31                | pcs00380    |
| Propanoate metabolism                               | 8            | 22                | pcs00640    |
| Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 7         | 19                | pcs00563    |
| Valine, leucine and isoleucine biosynthesis         | 6            | 16                | pcs00290    |
| Porphyrin and chlorophyll metabolism                | 6            | 16                | pcs00860    |
| Peroxisome                                          | 14           | 42                | pcs04146    |
| RNA degradation                                      | 16           | 49                | pcs03018    |
| Phagosome                                           | 11           | 33                | pcs04145    |
| Arginine biosynthesis                               | 6            | 17                | pcs00220    |

| Pathway                                             | Input number | Background number | Pathway ID  |
|-----------------------------------------------------|--------------|-------------------|-------------|
| Glucose, serine and threonine metabolism            | 11           | 38                | pcs00260    |
| beta-Alanine metabolism                              | 8            | 21                | pcs00410    |
| Valine, leucine and isoleucine degradation           | 9            | 32                | pcs00280    |
| Glyoxylate and dicarboxylate metabolism              | 8            | 36                | pcs00630    |
| Biosynthesis of secondary metabolites               | 31           | 302               | pcs01110    |
| Amino sugar and nucleotide sugar metabolism         | 9            | 48                | pcs00520    |
| Tyrosine metabolism                                 | 7            | 31                | pcs00350    |
| Cysteine and methionine metabolism                  | 8            | 43                | pcs00270    |
| Glutathione metabolism                              | 6            | 26                | pcs00480    |
| Biosynthesis of unsaturated fatty acids             | 4            | 12                | pcs01040    |
| Alanine, aspartate and glutamate metabolism         | 6            | 29                | pcs00250    |
| Fatty acid metabolism                               | 6            | 29                | pcs01212    |
| Propanoate metabolism                               | 5            | 22                | pcs00640    |
| Arginine and proline metabolism                     | 6            | 32                | pcs00330    |
| Protein processing in endoplasmic reticulum         | 9            | 70                | pcs04141    |
| Phenylalanine metabolism                            | 4            | 22                | pcs00060    |
| Carbon metabolism                                   | 11           | 103               | pcs01200    |
| Biosynthesis of antibiotics                         | 20           | 231               | pcs01130    |
| Fructose and mannose metabolism                     | 4            | 28                | pcs00051    |
| Peroxisome                                          | 5            | 42                | pcs04148    |
| Tryptophan metabolism                               | 4            | 31                | pcs00380    |
| Fatty acid degradation                              | 3            | 20                | pcs00071    |
| Steroid biosynthesis                                | 3            | 21                | pcs00100    |
| Fatty acid biosynthesis                             | 2            | 12                | pcs00061    |
| Ether lipid metabolism                               | 2            | 12                | pcs00565    |
| Histidine metabolism                                | 2            | 13                | pcs00340    |
| Various types of N-glycan biosynthesis              | 3            | 25                | pcs00513    |
| Inositol phosphate metabolism                       | 3            | 26                | pcs00562    |
| Glycerocephospholipid metabolism                    | 4            | 39                | pcs00564    |
| N-Glycan biosynthesis                               | 3            | 27                | pcs00510    |
| Nitrogen metabolism                                 | 2            | 15                | pcs00910    |
| Valine, leucine and isoleucine biosynthesis         | 2            | 16                | pcs00290    |
| Butanoate metabolism                                | 2            | 17                | pcs00860    |
| Cyanoamino acid metabolism                          | 2            | 18                | pcs00460    |
| Methane metabolism                                  | 2            | 19                | pcs00680    |
| Lysine degradation                                  | 2            | 20                | pcs00310    |
| Glycerolipid metabolism                             | 2            | 23                | pcs00561    |

(Continued)
of the significant enrichment terms were presented in the Supplementary Table S4. In biological process, main functional terms were cellular homeostasis, cell redox homeostasis, homeostatic process, organic acid catabolic process, carboxylic acid catabolic process and regulation of biological quality among others; cellular component mainly enriched in terms of integral component of membrane, intrinsic component of membrane, membrane part, plasma membrane, endoplasmic reticulum and endoplasmic reticulum membrane among others. And significant enrichment terms in molecular function were cofactor binding, coenzyme binding, hydrolase activity, hydrolyzing O-glycosyl compounds and hydrolase activity, acting on glycosyl bonds.

**Enrichment Analysis of KEGG Pathways**

For KEGG analysis, the DEGs were mapped to 91 and 79 KEGG pathway in samples treated with C$_{12}$O$_3$TR for 2 and 12 h, respectively. The 50 most enriched pathways were shown in Table 2. The two groups had the same expression patterns, mainly in cell wall, cell membrane, genetic information and energy metabolic pathways. In detail, the pathways which were associated with cell wall were fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism; cell membrane metabolic pathways were mostly sphingolipid metabolism, glycerophospholipid metabolism, glycerolipid metabolism, inositol phosphate metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, ether lipid metabolism, fatty acid biosynthesis, biosynthesis of unsaturated fatty acids, steroid biosynthesis, fatty acid metabolism and fatty acid degradation; genetic information pathways were ribosome, spliceosome, SNARE interactions in vesicular transport (The transport membrane bubble must fuse with the target membrane during transport to achieve the purpose of transport. A model to explain the mechanism at the molecular level is known as the SNARE hypothesis.), RNA degradation, protein processing in endoplasmic reticulum, proteasome and protein export; and the pathways which were related to energy metabolism were citrate cycle (TCA cycle), sulfur metabolism and nitrogen metabolism. In addition, there were difference between the expression patterns of 2 and 12 h. The genes of ribosome, spliceosome and RNA degradation which were related to genetic information processing were only differentially expressed in 2 h. Interestingly, the genes of protein processing in endoplasmic reticulum, proteasome and protein export which were also in connection with genetic information processing were only differentially expressed in 12 h.

**Verification of the Expression Level of Candidate DEGs**

A total of ten genes which were related to cell membrane and cell wall metabolisms and were simultaneous differently expressed in both 2 and 12 h were selected to validate the RNA-Seq results (Supplementary Table S1). The results of qRT-PCR experiments revealed that the genes showed the same expression profile as the RNA-Seq data, and then confirmed the reliability of the RNA-Seq data (Figure 4).
Effect of C$_{12}$O$_3$TR on the Cell Wall Integrity of *P. digitatum*

Figure 6 exhibited the effect of C$_{12}$O$_3$TR on the cell wall integrity of *P. digitatum*. When mycelia were stained with CFW for 0, 2, and 12 h without C$_{12}$O$_3$TR, almost every septa were visible with bright blue fluorescent lines because of the high chitin content therein (Figures 5A–C). However, the weaker and fewer blue fluorescence septa were observed in C$_{12}$O$_3$TR treated groups than that in control group when treated with the same time (Figures 5D–F). And the number of blue fluorescent septa gradually decreased along with the C$_{12}$O$_3$TR treatment time. Almost no blue fluorescence septa could be observed in after 12 h of C$_{12}$O$_3$TR treatment groups (Figure 5F).

Effect of C$_{12}$O$_3$TR on Cell Wall Related Enzymes Activities in *P. digitatum*

The influence of C$_{12}$O$_3$TR on enzyme activities related to cell wall metabolism of *P. digitatum* were shown in Figure 6. The
β-1, 3-glucan synthase is the vital enzyme which controlled the synthesis of glucan in cell wall. In this study, the β-1, 3-glucan synthase activity was decreased along with the treatment time (Figure 6A). C12O3TR significantly reduced the β-1, 3-glucan synthase activity which was 0.83 ± 0.04 mg/(g·h) when treated with C12O3TR for 12 h, while the activity was still 2.91 ± 0.01 mg/(g·h) in control group.

The chitinase is the vital enzyme which effectively catalytic hydrolysis of chitin in cell wall. For chitinase activity, C12O3TR treated group showed higher chitinase activity than control group (P < 0.05), and the chitinase activity also increased with the C12O3TR treatment time (Figure 6B). At 12 h, the chitinase activity in C12O3TR treatment was 37.74 ± 1.79 μg/(g·h), which was significantly higher than that in control group [21.79 ± 1.93 μg/(g·h)].

AKP is produced in the cytoplasm and leaked into the periplasmic space. Generally, AKP releases from fungal cells with impaired cell wall permeability. In our study, C12O3TR also increased the AKP activity in P. digitatum (Figure 6C). After 2 h of treatment, the AKP activity in C12O3TR group was 0.08 ± 0.00 μmol/(g·min), yet it was 0.05 ± 0.01 μmol/(g·min) in control group. This change became more evident when treated with longer time (P < 0.05). At 12 h, the extracellular AKP activity in the C12O3TR treatment was 0.77 ± 0.07 μmol/(g·min), which was significantly higher than that in control group [0.23 ± 0.02 μmol/(g·min)].

**DISCUSSION**

Our previous study found that peptide C12O3TR was effective to inhibit the growth of P. digitatum. Therefore C12O3TR was useful to control the green mold on citrus fruit (Li et al., 2019). The present works aimed to further investigate the possibly antifungal mechanism of C12O3TR against P. digitatum by transcriptomic profile determination through RNA-Seq analysis. Our results showed that C12O3TR significantly influenced a large number of gene expression and metabolic processes. The expression patterns of DEGs at 2 and 12 h were similar, which included the metabolic processes in cell wall, cell membrane, genetic information and energy. However, the expression of pathways, which were related to genetic information processing, were different in 2 and 12 h. The difference shown that the effect of C12O3TR on P. digitatum was different with the treat time owing to differential cellular statuses. This result was similar with the effect of essential oil decanal on the postharvest fungal pathogen Penicillium expansum in different time (Zhou T. et al., 2018).

Cell membrane plays an important role in maintaining the cell viability because it is a barrier that separates the cell from its surroundings, and is a channel for exchanging substances and energy between the cell and the surrounding environment (Shao et al., 2013; Tao et al., 2014). Its integrity is highly related to many metabolic processes. In this study, C12O3TR affected multiple cell membrane related metabolic processes, such as fatty acid biosynthesis, biosynthesis of unsaturated fatty acids, steroid biosynthesis, fatty acid metabolism and fatty acid degradation among others (Table 2). These results indicated that C12O3TR disrupted the normal metabolic processes of the cell membrane, and finally damaged the cell member integrity. In addition, we have demonstrated that C12O3TR could enhance the membrane permeabilization of P. digitatum by using fluorescence microscopy in our previous study (Li et al., 2019). These results were similar with most studies which...
researched the mechanism of AMPs. For example, peptide MAF-1A could inhibit the growth of *Candida albicans* by changing the normal expression of genes which encoded ergosterol metabolism and fatty acid biosynthesis. And these pathways were related to the metabolic processes of cell membrane (Wang et al., 2017).

The cell wall is mainly composed by mannose glycoprotein, β-glucan and chitin. The RNA-Seq results also indicated that the cell wall of *P. digitatum* was affected by C$_{12}$O$_3$TR, due to the changes in fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism (OuYang et al., 2016; Wang et al., 2018c). This finding was further confirmed by the changes of cell wall related enzymes activities, including β-1, 3-glucan synthase, chitinase and AKP (Figure 6). Similarly, many AMPs were found to be powerful to inhibit fungi growth by destroying the cell wall structure (Le et al., 2016; Wang et al., 2017). Cell wall is important in sustaining cell morphology and protecting cell against life threatening environmental conditions (Bowman and Free, 2006; Ruiz-Herrera et al., 2006). Chitin is one of the major macromolecule in the cell wall of filamentous fungi, and is very useful for the fungal development and pathogenicity (Klis et al., 2009). The CFW staining observation proved the effect of C$_{12}$O$_3$TR on the cell wall (Figure 5). CFW was widely used to determine the integrity of cell wall because of its preferential bounds to the chitin containing regions (Lewtak et al., 2014). In this study, the fewer and weaker blue fluorescence septa were observed under C$_{12}$O$_3$TR stress (Figure 5), indicating that the chitin content in the cell wall was influenced by C$_{12}$O$_3$TR treatment. This result was similar with the effect of antifungal proteins on cell wall chitin (Gandía et al., 2019). It was reported that the changes of chitin content was related to the activity of related enzymes, such as β-1, 3-glucan synthase and chitinase (OuYang et al., 2019). The present results showed that C$_{12}$O$_3$TR stress decreased β-1, 3-glucan synthase activity of *P. digitatum* but increased the chitinase activity (Figures 6A,B). The decrease of β-1, 3-glucan synthase activity lead to the decreased in the synthesis of glucan, and glucan was an essential precursor of chitin, therefore the chitin content was also reduced. On the other hand, chitinase was effective to catalyze the hydrolysis of chitin, thus the increase of chitinase activity also resulted in a sharp decline in chitin content. Moreover, AKP was an enzyme which was produced in the cytoplasm and usually located in the periplasmic space. If the permeability of cell wall was impaired, AKP would be released from fungal cells to the intercellular spaces (Yang et al., 2016). The increase of AKP activity further confirmed the damaged of the cell wall integrity by C$_{12}$O$_3$TR (Figure 6C).

Meanwhile, the metabolic processes of genetic information and energy were also found to be affected by C$_{12}$O$_3$TR stress (Table 2). But the effect on genetic information and energy metabolic process were only examined at transcriptional level. Further investigations were required to determine the anti-*P. digitatum* key mechanisms.

**CONCLUSION**

Overall, the results from this study at transcriptional level revealed that C$_{12}$O$_3$TR was effective to inhibit *P. digitatum* growth through complex influences on *P. digitatum* metabolisms. And this study also observed the impairment on cell wall formation at superficial level. Further studies are still required to...
investigate the key anti-fungal mechanisms of C12O3TR against P. digitatum especially the genetic information and energy related metabolic processes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: http://bigd.big.ac.cn/gsa/s/oUJ31ulZ.

AUTHOR CONTRIBUTIONS

KZ conceived and supervised the project. XL, GF, and WW designed the experiments and performed most of the experiments. XL analyzed the data and wrote the manuscript. LD and LY gave advises and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.574882/full#supplementary-material

TABLE S1 | The primer sequence and log2 (foldchange) value of DEGs.

TABLE S2 | Profile of the objective transcriptome sequence data.

TABLE S3 | The significant enrichment terms in the groups of C2 VS C12O3TR2.

TABLE S4 | The significant enrichment terms in the groups of C12 VS C12O3TR12.
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Conflict of Interest: 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.

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