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Full Length Article

Transcriptome Analysis of Korla Fragrant Pear Reveals a Comprehensive Signaling Network in Response to *Alternaria alternata* Infection

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Abstract

Blackhead caused by *Alternaria alternata* is a fatal necrotrophic fungal that affects Korla fragrant pear. To date, little is known at the molecular level about the defense response of pear to blackhead disease and the pathogenic mechanism of *A. alternata* infection. To investigate the specific host-pathogen interaction between *A. alternata* and pear, we examined the accumulation of host-responsive mRNAs using RNA-seq technology. A total of 25,877 differentially expressed genes (DEGs) were identified. Further analysis revealed that the DEGs mainly participate in plant cell wall integrity, plant hormone pathways, plant-pathogen interactions and the defense response (transcription factors, defense-related proteins). Most of the DEGs involved in the plant hormone, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) pathways, as well as defense-related proteins, were significantly up-regulated. In addition, DEGs encoding enzymes involved in cutin and wax synthesis and most transcription factors are significantly down-regulated. Based on these results, we speculate that these pathways play important roles in the response of pear to *A. alternata*. This study has presented new insights into the molecular mechanisms that regulate the response of pear fruits to *A. alternata* infection.

Keywords: *Alternaria alternata*; Blackhead disease; Defense response; Infection; Korla fragrant pear; RNA-seq

Introduction

Korla fragrant pear (*Pyrus sinkiangensis* Yu) is a traditional high-quality fruit grown in Xinjiang province, China (Ma *et al.* 2019). It is popular with consumers because of the thin exocarp, crispy flesh, high juice and sugar content and pleasant rich fragrance (Chen *et al.* 2020). At present, Korla fragrant pear is exported to many countries around the world and has high commercial value on the international market due to its special flavour and nutritional qualities (Tian *et al.* 2014). Unfortunately, Korla fragrant pear is prone to diseases such as blackhead disease, scab, powdery mildew, brown spots, fire blight and other fungal diseases during long-distance transportation or long-term storage (Cheng *et al.* 2019). Among these diseases, blackhead disease mainly occurs during storage and its incidence rate can be as high as 10%, making blackhead the main storage disease of Korla fragrant pears.

Blackhead disease caused by *Alternaria alternata* is a fatal necrotrophic fungal disease that affects Korla fragrant pear quality and production (Chen *et al.* 2019). At the early stage of fruit infection, the lesions first appear at the calyx end of the pear, the peel in the diseased area turns brown-black, the flesh appears as a light brown honeycomb and the uninfected pulp tissues look good but taste slightly bitter. As the disease development progresses, the surface of the fruit collapses and produces a sticky black juice-like substance. A distinct boundary appears between the lesion and the internal good flesh and a white mold layer grows on the peel that leads to a decline in pear fruit quality. At present, chemical fungicides are the main method used to inhibit fungal disease (Dalcero *et al.* 1996). However, long-term and large-scale use of chemical fungicides may lead to strong resistance in *A. alternata* and have an adverse effect on the environment (Ma and Michailides 2005). Therefore, an improved description of the host-pathogen interaction and the pear defense response against *A. alternata* infection will help provide a reference for understanding the causes of blackhead disease and biological control of the pathogen.

With the rapid development of molecular biology and associated bioinformatics tools, considerable progress has been made in understanding plant-pathogen interactions. RNA-seq technology is an important tool to explore complex biological processes (Marguerat and Bähler 2010). At present, RNA-seq technology has been frequently used to study the interaction between plants and pathogens in

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horticultural crops. For example, RNA-seq analysis have provided valuable information about changes in gene expression in host-pathogen interactions involving apple and *A. alternata* (Zhu *et al.* 2017), apple and powdery mildew fungi (Tian *et al.* 2019), Callery pear and *A. alternata* (Kan *et al.* 2017). In this study, RNA-seq was used to explore the transcriptomic profiles of Korla pears in response to fungal infection. Our objective is to explore the potential causes of susceptibility of pears to *A. alternata* infection at the molecular level, so as to provide support for the development of new storage and preservation technologies for pear fruits.

Materials and Methods

Plant materials, *A. alternata* culture and inoculation procedures

Five-year-old Korla fragrant pear plants were grown in a pear orchard located in Xinjiang province, China. This study cultured the *A. alternata* fungus on potato dextrose agar (PDA; 20 g dextrose, 200 g potato extract and 20 g agar in 1 L of water) medium at 28°C in the dark. After 5 days, conidia were collected in ~20 mL of distilled sterile water per plate by swirling gently to detach the conidia. The conidial suspension was then centrifuged and the conidia were diluted until reaching the concentration of 1×10^5 conidia/mL. The surface of pears was disinfected with 75% alcohol and then rinsed in sterile distilled water for 3 times. After air drying, 3 holes were punched in the surface of the pears (diameter = 1 mm; deep = 1 mm) and 20 μL of the conidial suspension was introduced in these holes using a pipette. The control group (T0) was inoculated with sterile water. There were four experimental pear groups, each consisting of 30 inoculated fruits. The fruits were incubated in sterile plastic chambers at 25°C under a 14 h light/10 h dark cycle and fruit tissues around the wounded sites (1 cm deep x 1 cm diameter)) were taken at 24, 72 and 96 hours as experimental groups T1, T3 and T5, respectively. After flash freezing in liquid nitrogen, the fruit samples were stored at -80°C for later use during the RNA-seq experiments. Each stage involved three parallel fruit samples which were picked at the same time and represented three biological replicate samples, respectively.

Total RNA extraction, library construction and sequencing

The cetyltrimethyl ammonium bromide (CTAB)-based method was used to isolate total RNA and to improve the RNA isolation, column purification was performed using a RNAeasy Plant Mini kit produced by Qiagen (Germany). The three biological replicates at different stages of infection were combined to construct a complementary DNA (cDNA) library. Next, magnetic beads were used to purify the products and oligo (dT) magnetic beads were used to enrich mRNAs. Then short fragments of ~200 bases were prepared by mixing the mRNAs were with fragmentation buffer and they were used as templates to synthesize first-strand cDNA and random hexamer-primers. Second-strand cDNAs were synthesized using Buffer, dNTPs, RNase H and DNA polymerase I. The short two-stranded cDNAs were subject to purification *via* a QiaQuick PCR extraction kit. After repair, the cDNA fragment ends and poly (A) tails were connected to the Illumina sequencing adapters. Fragments of different size ranges were separately recovered by agarose gel electrophoresis and fragments of the appropriate sizes were enriched by PCR amplification. The constructed libraries were subject to sequencing using an n Illumina HiSeq™ platform.

RNA-seq data analysis

We converted the raw image data obtained from the sequencing instrument into raw sequence reads and saved it in FASTQ file format. Raw data were used so that high-quality clear reads for subsequent analysis can be obtained and they were trimmed to remove the sequencing adapters. Reads consisting only of adapters, those with > 10% Ns (unknown bases) and low-quality reads in which the percentage of bases with phred quality scores $Q \leq 20$ was >50% were removed from the data sets.

The relative gene expression level was calculated using Fragments Per Kilobase of transcript per Million mapped reads (FPKM). The criteria for identifying differentially expressed genes (DEGs) were a False Discovery Rate (FDR) ≤ 0.05 and the absolute value of |fold-change (FC)| ≥ 1. Annotation of proteins encoded by the DEGs was performed by GO functional classification using the Blast2GO program. We then used WEGO software to analyze the significant functional enrichment of the DEGs. Finally, the GO terms that showed significant enrichment in the DEGs was founded using the hyper-geometric test with $P \leq 0.05$ as the threshold. We also used the DEGs as queries to search the KEGG database to analyze pathway enrichment using the same criteria described above.

qRT-PCR verification

We selected 10 random DEGs for qRT-PCR to analyze and verify the RNA-seq data. Based on the sequence information of the selected DEGs, we designed gene-specific primers for qRT-PCR using the Primer Express software. Total RNA samples (1 μg) from pears were used for cDNA synthesis by reverse transcription using the FastQuant RT Kit (Beijing, China) as directed by the manufacturer. The qRT-PCR assays were used on an ABI StepOnePlus Real-Time PCR System (ABI, U.S.A.) and the reaction mixtures (20 μL) contained 0.4 μL of the forward and reverse PCR primers (10 μM), 10 μL of qPCR Master Mix and 4 μL template cDNA. The amplification program

was 95°C for 90 s and then 40 cycles of 95°C for 5 s, 60°C for 15 s and 72°C for 20 s. Internal control on normalization of gene expression was performed using the pear *Actin* gene (*Actin2/7*) and the $2^{-\triangle\triangle Ct}$ method was used to calculate the relative expression level of selected unigenes. Three independent biological replicates were selected for each sample and Table 1 shows all the primers used.

Results

Microscopic analysis of Korla fragrant pears infected with *A. alternata*

By observing the symptoms of *A. alternata*-infected pears at different time points, we found that a few spores and hyphae were newly produced on the surface of the peel at 1 day post infection (dpi) (Fig. 1A). At 3 dpi, the spotted brown lesions on the surface of the pears were stained with white mycelia and the early-stage symptoms of blackheads became apparent (Fig. 1B). After 5 dpi, noticeable lesions appeared in the inoculated sites and were covered with large areas of white hyphae accompanied by softening and depression of the peel and pulp tissue (Fig. 1C).

To further determine the optimal sampling time and observe the process of *A. alternata* infection of fragrant pears, scanning electron microscopy and lactophenol trypan blue staining were used to observe the process of spore germination and hyphal production and growth. Microscopic observation showed that the spores start to germinate at 1 d and formed embryo tubes. Three days after inoculation, the germ tubes were further extended and the hyphae showed obvious growth and adhered to the surface of the peel. On the fifth day following inoculation, the surface of the peel including the inoculation site was covered with white hyphae and the hyphae had invaded the pulp tissue, causing symptoms such as brown lesions, softening and surface depression (Fig. 1D–I).

RNA-seq data and DEG profiles in response to *A. alternata* infection

Changes in transcript levels in comparisons of the non-inoculated control and the inoculated groups (T0 *vs*. T1, T0 *vs*. T3 and T0 *vs*. T5) were determined in an RNA-seq experiment. We obtained a total of 196.25 million raw 300 bp paired end sequencing reads. After filtering out the low-quality reads, 191.83 million clean reads remained. Of these, 91.35 million (48.10%) were mapped to the pear genome reference sequence and 87.20 million (44.09%) of the clean reads mapped uniquely to the pear genome reference sequence. Based on these mapped reads, the expression levels of 13,621 DEGs were calculated using the FPKM method.

Gene expression between the *A. alternata* infected groups (T1, T3 and T5) and the control group (T0) were compared. The results identified 5,877, 7,970 and 11,485 DEGs at 1, 3 and 5 d, respectively (Fig. 2A). Among the DEGs, there was no significant difference in the number of up-regulated genes, but the number of down-regulated genes increased gradually with the severity of infection. For example, there were 3,499, 3,580 and 3,503 up-regulated genes at 1, 3 and 5 d, but 2,378, 4,390 and 7,987 genes were down-regulated at these three time points (Fig. 2B). Most of the DEGs were detected at 5 dpi, which suggests that this is a critical stage in the host response to *A. alternata* infection in pear fruits.

In addition, we visualized the number of DEGs from the different comparisons in a Venn diagram, which clearly shows that both unique DEGs and shared DEGs are present in the different groups (Fig. 2C). Among them, 3,958 DEGs were shared among the three pairwise comparisons and the T0 *vs*. T1 comparison shared 4,494 and 4,308 DEGs with the T0 *vs*. T3 and T0 *vs*. T5 comparisons, respectively. Moreover, there were 7,415 DEGs shared between the T0 *vs*. T3 and T0 *vs*. T5 comparisons, which is significantly higher than in the above two comparisons.

Functional annotation of the DEGs

To explore the function of the DEGs, a GO analysis was carried out by mapping them to three major GO categories, biological process, molecular function and cellular component when the corrected *P*-value was < 0.05. In the biological process category, the GO terms "metabolic process" (2,577 DEGs), "cellular process" (2,184 DEGs) and "single-organism process" (1,699 DEGs) were the most enriched groups. In the molecular function category, "catalytic activity" (2,544 DEGs) and "binding" (1,690 DEGs) were remarkably enriched and we found that these two terms were highly involved in the process of plant hormone signal transduction. For cell component, the significantly enriched terms were "cell" (983 DEGs), "cell part" (983 DEGs), "macromolecular complex" (305 DEGs), "membrane" (784 DEGs), "membrane part" (616 DEGs), "organelle" (654 DEGs) and "organelle parts" (336 DEGs) (Fig. 3).

To identify pathways that play key roles in plant-pathogen interactions, 8,512 DEGs were further assigned to 45 different categories by KEGG pathway analysis. The results showed that several secondary metabolite pathways involved in defense, such as those involved in synthesis of terpenoid derivatives and flavonoids, were enriched. These pathways included "tropane, piperidine and pyridine alkaloid biosynthesis", "ubiquinone and other terpenoid-quinone biosynthesis", "monoterpenoid biosynthesis" and "terpenoid backbone biosynthesis" (Table 2). Moreover, the pathways for defense signaling transduction and plant pathogen recognition, which are related to "plant hormone signal transduction", "cutin, suberine and wax biosynthesis" and "plant-pathogen interaction" were also enriched. However, the timing of gene induction was different. For instance, the pathways "plant hormone signal transduction" and "cutin,

Table 1: Primers used for qPCR

Gene ID	Gene Name	Forward primer (5'--3')	Reverse primer (5'--3')
-	Actin2/7	CTCCCAGGGCTGTGTTTCCTA	CTCCATGTCATCCCAGTTGCT
LOC103936633	EMB1144	CTGTGCCGATGGTGGAAG	AGTTGCTGCCTCCGCT
LOC103939953	PLD1	CCCCCTTCCATTCACTTTTCAG	ACCACCTTGCTTTCTCCACC
LOC103943393	IMP1	ATTCGCTTGCTCAGTTCCTCT	CCTTATCAGTTTCCGTGACCAG
LOC103948139	FBA3	GACGAACTCCTCCTAACTGCC	CTTCCCATCGGTTGTAGACTG
LOC103951141	ADH	CACCACCACAGGCAAATGAAG	TGTCACGCCCTCACCAATAC
LOC103951803	At5g47720	ATGGGTGGTTTTCTGGGTGC	CTGTATGCTCTGTGCTGCGA
LOC103957712	Cht5	AACAGGTCAAGTTTCGGTGG	CAAGAAAAGATTGCCGTGTGTAG
LOC103958948	FQR1	TTGCTGAGGCTGATGGGATA	GCTGTCAAGGGGGTAGTCTC
LOC103965128	PDC4	GGGACGCACAGGATTCTTCA	CCTGATAGCAAGTCACGGTCTG
LOC103966218	DPE2	GTCTGCTACTGAGCCCTGTC	ACATTTGAAGCCCTTTGGAAC

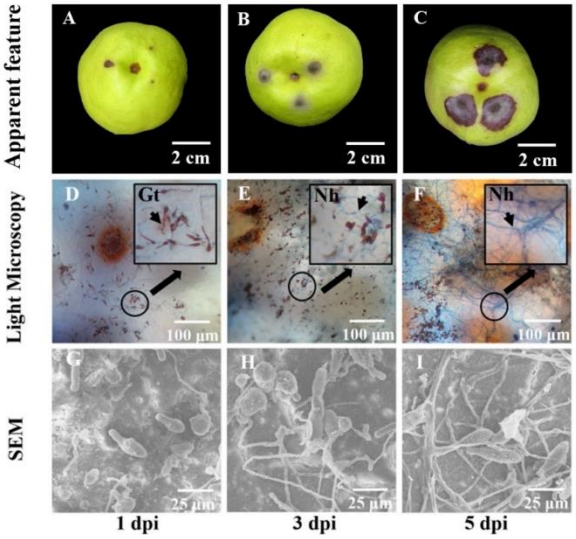

Fig. 1: Symptoms of *A. alternata* infection of Korla fragrant pear. Note: (**A**-**C**) Changes in symptoms of Korla fragrant pear after *A. alternata* infection; (**D**-**F**) Lactol trypan blue staining of pear epidermis inoculated with *A. alternate*; (**D**) The spores gradually germinate and form germ tubes; (**E**) Germ tube grows and produces a little mycelium; (**F**) White mycelium further invaded and expanded, producing obvious lesions at the inoculation site. GT, germ tube; Nh, New hypha; (**G**-**I**) Scanning Electron Microscopy observation of infection of Korla fragrant pear by *A. alternata*

suberine and wax biosynthesis" were induced earlier on the first day, while the "plant-pathogen interaction" pathway was induced later on the 3rd and 5th days.

DEGs involved in plant cell wall integrity

The plant cell wall is an important physical barrier against pathogen infection and is the main composition of the monitoring system in the plant innate immune system. There are many genes involved in plant cell wall biosynthesis, including *HTH* (Hothead) (Kurdyukov *et al.* 2006), *WSD* (Wax-ester synthase/ diacylglycerol o-acyltransferase) (Li *et al.* 2008), *GPAT* (Glycerol-3-phosphate acyltransferase) (Gidda *et al.* 2009), *CER* (Eceriferum) (Aarts *et al.* 1995), *PME* (Pectin methylesterase), (Bethke *et al.* 2014) and *XTH* (xyloglucan endotransglycosylase/hydrolase) (Rose *et al.* 2002). In this study, the RNA-seq data showed that expression of two *GPAT6* (LOC103931179; LOC103949465), one *HTH1* (LOC103929758), two *CER1* (LOC103932050; LOC103942391), three *WSD1* (LOC103928845; LOC103941920; LOC103961411) and 13 *XTH* genes was down-regulated during the infection process (Table 2). In addition, we found that expression of 10 *PME* genes showed an upward trend after plants were infected with *A. alternata* (Fig. 4).

DEGs involved in plant hormone signaling pathways

Plant hormones, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and brassionosteroids (BRs), are critical factors involved in the plant disease defense response (Tian *et al.* 2019). To identify DEGs associated with hormonal responses in pears infected with *A. alternata*, we analyzed the hormone signal transduction pathways. In this study, three DEGs involved in SA signaling (*PR-1*, Pathogenesis-related proteins) and six DEGs in JA signaling (*JAZ*, Jasmonate zim domain) were up-regulated (Fig. 5A- B); three DEGs involved in ET signaling encoding ERF1/2 (Ethylene response factor 1/2)

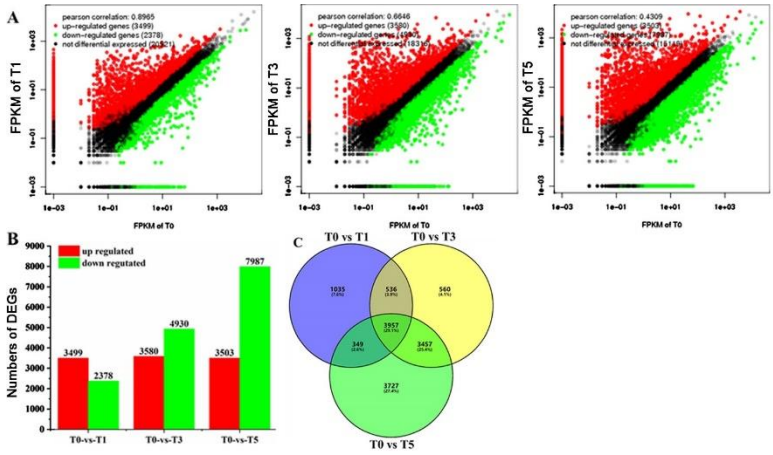

Fig. 2: DEGs between samples. (**A**) Scattered plot of differential expression. (**B**) Numbers of DEGs compared between two samples (T0 *vs.* T 1 dpi, T0 *vs.* T3 dpi, T0 *vs.* T5 dpi and with T0 dpi as the control). Red shows up-regulated, green shows down-regulated. (**C**) Venn diagram analysis of the DEGs in Korla fragrant pear after *A. alternata* infection

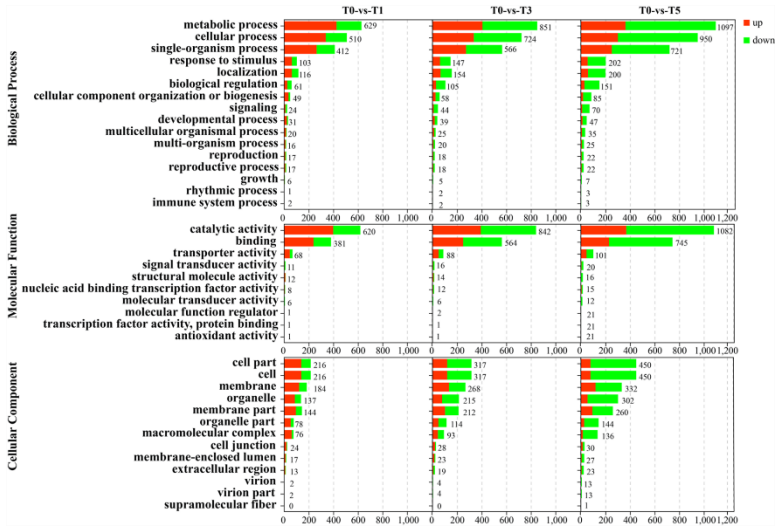

Fig. 3: GO categories of DEGs in Korla fragrant pear in response to *A. alternata* infection (red for up-regulated, green for down-regulated)

were up-regulated in the three stages (Fig. 5C); In addition, the genes involved in the ABA signal perception and transduction pathway, for example PYR/PYL (pyrabactin resistance1/PYR1-like) were up-regulated (Fig. 5D); DEGs involved in the BR-response, such as *BRI1* (BRI-associated receptor kinase 1), *BSK* (brassionosteroid insensive) and *TCH4* (*X*yloglucan endotransglucosylase, also known as Touch 4) that showed the same expression pattern were significantly upregulated at 5 dpi (Fig. 5E).

DEGs involved in plant-pathogen interactions

To identify DEGs associated with plant-pathogen interactions in the pears infected with *A. alternata*, we analyzed the plant-pathogen interaction pathways. In the present study, three DEGs encoding CDPK (calcium-dependent protein kinases) and two DEGs encoding Rbohs (respiratory burst oxidase homologue) were up-regulated in the three stages, inducing a hypersensitive response and cell wall reinforcement. In addition, 16 CNGC (cyclic nucleotide-gated ion channel) and 31 CaM/CML (calmodulin/cam-like) genes were regulated and among them, four DEGs encoding CNGC and six DEGs encoding CaM/CML were strongly up-regulated at 3 dpi and 5 dpi. Furthermore, four DEGs encoding RPM1 (RPM1-interacting protein) were also up-regulated at 3 and 5 dpi (Fig. 6). In addition two transcription factor genes, *WRKY33* and *WRKY29*, showed changes in expression; these genes encode proteins that participate in the MAPK (mitogen-activated protein kinase) signaling pathway and induce plant disease resistance.

DEGs encoding transcription factors (TFs)

TFs are important regulatory proteins that can regulate gene transcription by binding to specific sequence motifs in the

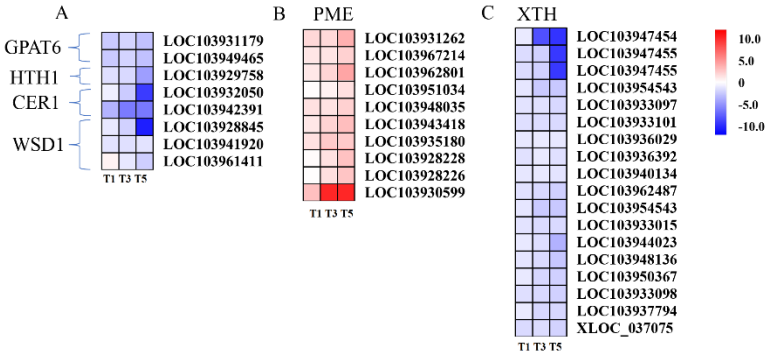

Fig. 4: Heatmaps of DEGs involved in plant cell wall integrity. The $\log_2^{|Foldchange|}$ was colored using OriginPro 2020 (red for up-regulated, green for down-regulated), each horizontal row represents a DEG with its gene ID and the vertical columns represent 1, 3 and 5 dpi from left to right. (**A**) DEGs related to stratum corneum; (**B**) Genes related with PME; (**C**) Genes related with XTH

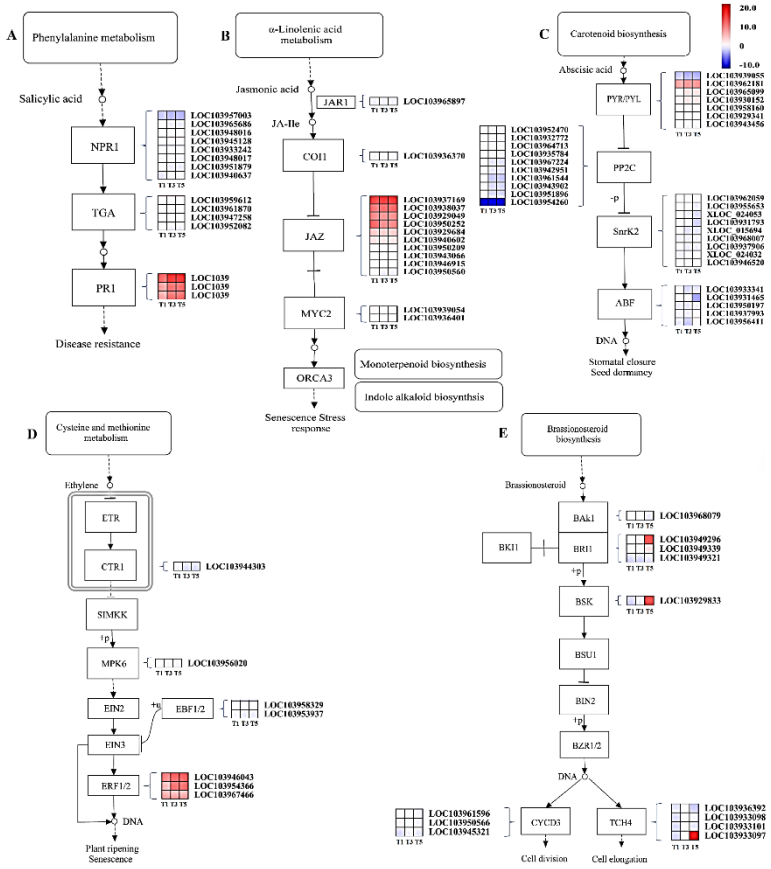

Fig. 5: Heatmaps of DEGs Involved in Plant Hormone Signaling Pathway. The $\log_2^{|Foldchange|}$ was colored using OriginPro 2020 (red for up-regulated, green for down-regulated), each horizontal row represents a DEG with its gene ID and the vertical columns represent 1, 3 and 5 dpi from left to right. (**A**) DEGs related to SA; (**B**) Genes related with JA; (**C**) Genes related with ET; (**D**) Genes related with ABA; (**E**) Genes related with BRs

promoter regions of downstream target genes (Vidhyasekaran 2016). In plants, WRKY, MYB, ERF, Hsfs, ZIP and NAC are all important transcriptional regulators of plant defense responses (Pandey and Somssich 2009; Dezar *et al.* 2011; Pajerowska-Mukhtar *et al.* 2012). In this study, several TF-encoding genes belonging to different families were either up- or down-regulated, including 54 *WRKY* genes, 29 *MYB* genes, 12 *ERF* genes, 17 *bHLH* genes, nine *ARF* genes, six *Hsf* genes, two *ZIP* genes and two *DOF* genes (Fig. 7). Interestingly, more DEGs were down-regulated than up-regulated in the MYB, ERF, ARF, Hsfs, ZIP and bHLH families. The expression of related TF genes in pear fruits after *A. alternata* infection is shown in Fig. 7.

Table 2: Significantly enriched KEGG pathway of DEGs in response to *A. alternata*

Pathway	Number of DEGs at each time point			Pathway ID
	T0 *vs*. T1	T0 *vs*. T3	T0 *vs*. T5	
Biosynthesis of secondary metabolites	396	556	693	ko01110
Terpenoid backbone biosynthesis	22	35	46	ko00900
Microbial metabolism in diverse environments	127	193	239	ko01120
Taurine and hypotaurine metabolism	13		17	ko00430
Biosynthesis of antibiotics	164	234	294	ko01130
Glycolysis / Gluconeogenesis	45	65	83	ko00010
Circadian rhythm - plant		28	38	ko04712
Photosynthesis			37	ko00195
Metabolic pathways	576	842	1080	ko01100
Carotenoid biosynthesis	17	23	31	ko00906
Pyruvate metabolism		46	63	ko00620
Porphyrin and chlorophyll metabolism			36	ko00860
alpha-Linolenic acid metabolism	35	40	43	ko00592
Regulation of autophagy		23	31	ko04140
Sulfur metabolism		25	30	ko00920
Biosynthesis of amino acids	92	128	155	ko01230
Folate biosynthesis		10	14	ko00790
Phenylalanine, tyrosine and tryptophan biosynthesis	27	29	37	ko00400
Vitamin B6 metabolism	10	10	14	ko00750
Sesquiterpenoid and triterpenoid biosynthesis		18	19	ko00909
Ubiquinone and other terpenoid-quinone biosynthesis			27	ko00130
Phenylalanine metabolism		22	28	ko00360
Pentose phosphate pathway		30	35	ko00030
Arginine and proline metabolism	23	30	36	ko00330
Tropane, piperidine and pyridine alkaloid biosynthesis	16	19	20	ko00960
Plant-pathogen interaction		97	114	ko04626
Steroid biosynthesis		22	27	ko00100
Tyrosine metabolism	22	25	27	ko00350
Selenocompound metabolism		10	12	ko00450
Monobactam biosynthesis	8	7	8	ko00261
Flavonoid biosynthesis	18	30		ko00941
Ribosome	152	161		ko03010
Galactose metabolism	21	38		ko00052
Isoquinoline alkaloid biosynthesis	13	15		ko00950
Monoterpenoid biosynthesis	8	10		ko00902
Cysteine and methionine metabolism	41	54		ko00270
Carbon fixation in photosynthetic organisms	30	39		ko00710
Pantothenate and CoA biosynthesis		21		ko00770
Carbon metabolism		118		ko01200
beta-Alanine metabolism	21	23		ko00410
Glutathione metabolism		44		ko00480
Fatty acid degradation	20			ko00071
Butanoate metabolism	11			ko00650
Cutin, suberine and wax biosynthesis	15			ko00073
Plant hormone signal transduction	87			ko04075

DEGs encoding defense-related proteins

Exposure to abiotic and biotic stresses triggers the expression of various defense-related proteins with antibacterial activity, which leads to other defense-related responses such as cell death, the HR and cell wall rigidification in plants. In our results, a number of DEGs encoding defense-related proteins belonging to different families were up-regulated at three detection time points; these included three *PR-1* genes, 10 *CHT* (chitinase) genes, six *TLP* (thaumatin-like protein) genes and seven *POD* (peroxidase) genes (Fig. 8). In contrast to the above four TF families, genes encoding HSP family proteins were significantly down-regulated (Fig. 8).

qRT-PCR verification

In order to verify the RNA-seq results, we randomly selected 10 DEGs from two time points (T0 and T3) of pear fruit infection for qRT-PCR analysis. The genes were shown to be either up- or down-regulated in the T0 *vs*. T3 comparison and our analysis showed that the qRT-PCR and RNA-seq data expression results were consistent and showed a significant positive correlation, verifying the accuracy and reliability of the RNA-seq data (Fig. 9).

Discussion

The Korla fragrant pear is a type of fruit that has both a high nutritional value and a high commercial value. However,

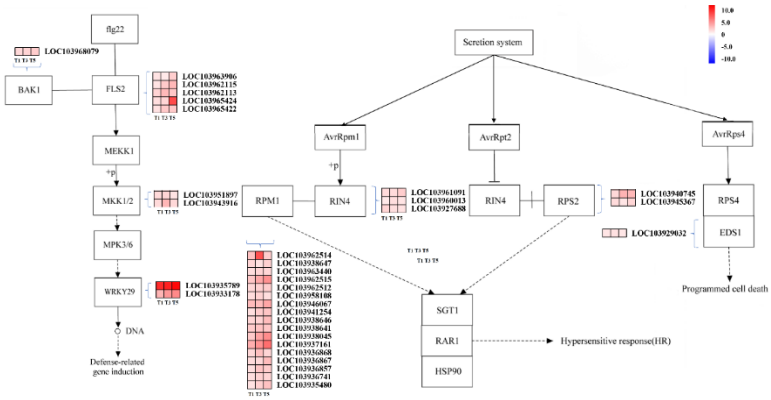

Fig. 6: Plant-Pathogen Interaction Pathway. The red for up-regulated, green for down-regulated (red for up-regulated, green for down-regulated), each horizontal row represents a DEG with its gene ID and the vertical columns represent 1, 3 and 5 dpi from left to right. (**A**) DEGs related to PTI; (**B**) DEGs related to ETI

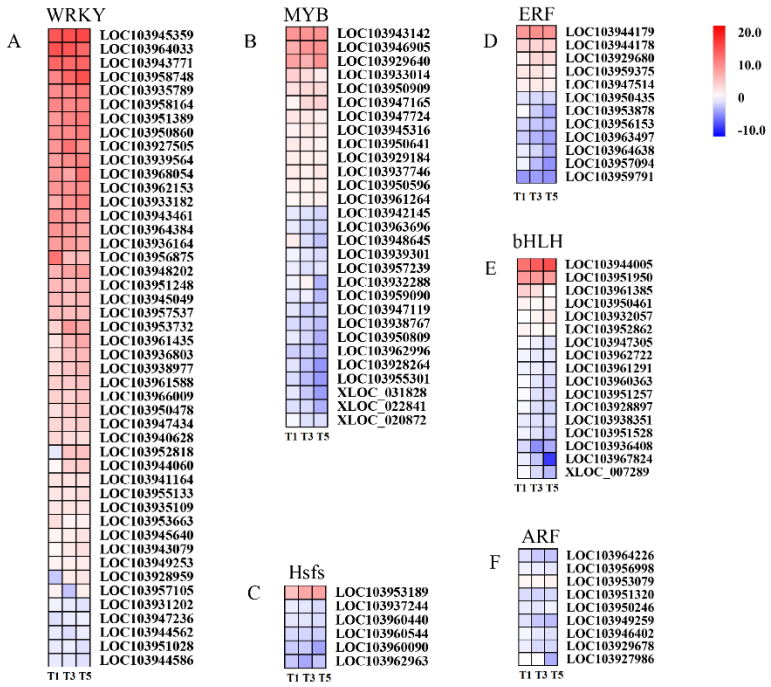

Fig. 7: Heatmaps of DEGs Involved in Transcription Factors. The $\log_2^{|Foldchange|}$ was colored using OriginPro 2020 (red for up-regulated, green for down-regulated), each horizontal row represents a DEG with its gene ID and the vertical columns represent 1, 3 and 5 dpi from left to right. (**A**) DEGs related to WRKY; (**B**) Genes related with MYB; (**C**) Genes related with Hsfs; (**D**) Genes related with ERF; (**E**) Genes related with bHLH. (**F**) Genes related with ARF

during storage, fragrant pears are readily infected by *A. alternata*, which leads to great losses. Therefore, we studied the transcriptome changes in fragrant pear in response to *A. alternata* infection in order to explore the potential reasons for the susceptibility of pears to blackhead disease at the molecular level and to provide support for the development of new storage technology for pear fruits. At present, the reference genome of Chinese white pear maintained by NCBI has a total of 42,194 genes (Wu *et al.* 2013). In this study, 25,877 (61.33%) genes were compared in all of the sample groups. The results of the enrichment analysis showed that the DEGs are mainly involved in metabolic pathways related to resistance, such as plant cell wall metabolic pathways, plant hormone signaling pathways, plant-pathogen interaction pathways and transcription factor regulation pathways, among others. Compared with other fruits, Korla fragrant pear has a number of unique characteristics. There is a dense wax layer on the surface of pear, which is very effective at maintaining fruit quality and controlling pathogenic microorganisms. The wax layer can further assist the plant cell wall and protect plant cells from microbial infection (Bellincampi *et al.* 2014). Many genes are involved in the biosynthesis of the plant cell wall and epidermal wax layer, including *HTH*, *WSD*, *GPAT*, *CER*,

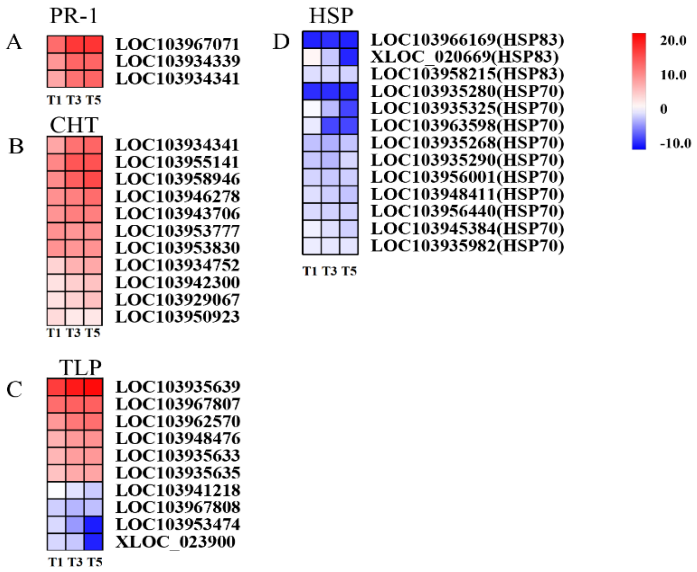

Fig. 8: Heatmaps of DEGs Involved in Defense-related proteins. The $\log2^{|Foldchange|}$ was colored using OriginPro 2020 (red for up-regulated, green for down-regulated), each horizontal row represents a DEG with its gene ID and the vertical columns represent 1, 3 and 5 dpi from left to right. (**A**) DEGs related to PR-1; (**B**) Genes related with chitinase; (**C**) Genes related with TLP; (**D**) Genes related with HSP

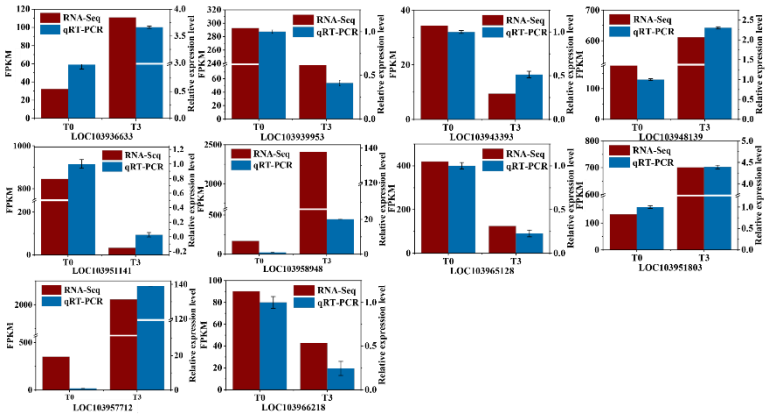

Fig. 9: Validation of RNA-seq data by qRT-PCR. 10 DEGs were selected for validation and they showed a similar tendency with RNA-Seq. Left vertical coordinate is RPKM of RNA-Seq; right vertical coordinate is relative expression level of qRT-PCR

PME and *XTH*. Our results show that the homologs of these genes in pear, *GPAT6*, *HTH1*, *CER1* and *WSD1* all showed down-regulated expression during the infection process. Previous studies have shown that the down-regulation of *HTH1* and *GPAT6* may have adverse effects on the formation of the stratum corneum (Ya *et al.* 2017), while the down-regulation of *CER1* and *WSD1* could affect the synthesis of the cuticular wax layer (Li *et al.* 2008). Therefore, the down-regulation of these genes in this study implies that the biosynthesis of the cuticle and wax is impaired in infected pears making it easier for *A. alternata* to penetrate, which may be the main reason why pears are more susceptible to pathogen infection. In addition, we found that 10 *PME* genes showed an upward expression trend after plants were infected with *A. alternata* (Fig. 4). It is well known that PME can catalyze the de-methylesterification of its pectin substrate. Therefore, the up-regulated expression of *PME* genes in this study may indicate that the degradation of plant cell wall components is accelerated.

Plant hormones are a general class of signaling molecules that play key regulatory roles in plant growth, development and defense responses. The complex interactions between different plant hormones *via* signaling pathways, called hormone crosstalk, can lead to changes in plant-specific metabolic pathways (Robert-Seilaniantz *et al.* 2011). The plant hormones usually involved in crosstalk are SA, JA, ET, ABA and BRs, which activate the corresponding defense reactions by regulating specific physiological responses, thereby preventing and resisting infection by pathogenic microorganisms (Bari and Jones 2009). In this study, genes involved in the SA (*PR-1*), JA (*JAZ*, *MYC2*), ABA (*PYR/PYL*), ET (*ERF1/2*) and BRs (*BRI1*, *BSK*, *TCH4*) signaling pathways were up-regulated

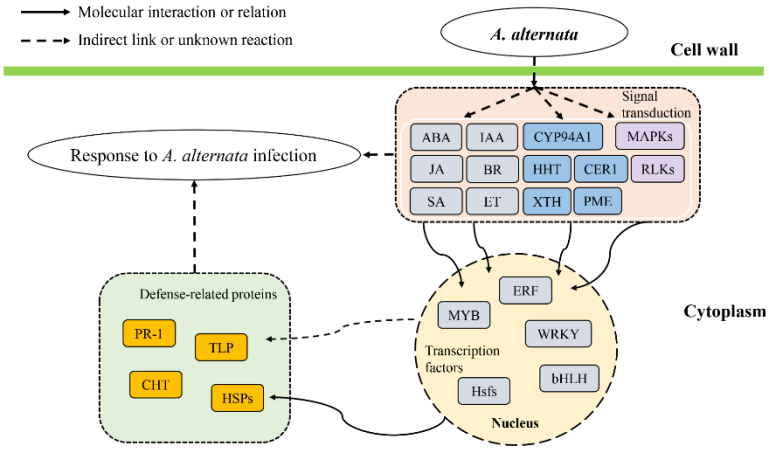

Fig. 10: Molecular network underlying the defense response to *A. alternata* in pear

when pears were infected with *A. alternata*. Some studies have shown that overexpression of *PR-1* may play a positive role in enhancing plant immunity to pathogens (Tian *et al.* 2019). We found that several DEGs associated with SA (*PR-1*) were significantly upregulated in response to *A. alternata* infection, suggesting that SA might participate in regulating the response to *A. alternata* in pears. Previous studies have shown that JA and ET are mainly involved in the defense response against necrotrophs (Zhu *et al.* 2017). In this experiment, the genes involved in JA (*JAZ*, *MYC2*) and ET (*ERF1/2*) signaling were up-regulated when the pears were infected by *A. alternata*, which is consistent with previous studies. ABA is an important regulator of the interaction between plants and pathogenic microorganisms (Laurens *et al.* 2017). Many studies have shown that ABA often interferes with defense signaling pathways such as the SA/JA/ET pathway, thus negatively regulating plant resistance (Zhu *et al.* 2017). In this study, the genes involved in ABA signaling pathways, for example *PP2C*, *SnrK2* and *ABF* were down-regulated while *PYR/PYL* were up-regulated in response to *A. alternata* infection, which revealed that the ABA signaling pathway was significantly inhibited after infection by *A. alternata*. This result is consistent with the response of apple leaves to *A. alternata* infection (Zhu *et al.* 2017). Finally, BRs play a complex and positive role in plant innate immunity (Tian *et al.* 2019). In this study, several DEGs associated with BR signaling were significantly upregulated in response to *A. alternata* infection, suggesting that BRs might participate in regulating the response to *A. alternata* infection in pear fruits.

In the process of resisting pathogen infection, plants mainly use two defense mechanisms. On the one hand, they trigger pathogen triggered immunity (PTI) by recognizing a broad range of pathogens with conserved molecular pattern on their surface and on the other hand, specific R genes that contain nucleotide binding site (NBS) and leucine-rice repeat (LRR) domains recognized specific pathogen proteins to trigger effector-triggered immunity (ETI) (Sun *et al.* 2013). In this study, we detected several genes involved in PTI and ETI that showed differential expression in response to *A. alternata* infection. BAK1 is one of the best studied receptor-like protein kinases (RLKs). A previous study has shown that flg22 induces BAK1 as a co-receptor and initiates immune signaling during the heterodimerization of FLS2 and BAK1 (Sun *et al.* 2013). In our research, the genes encoding BAK1 and FLS2 were up-regulated after inoculation, suggesting that BAK1 and FLS2 may promote the immune response in pear and lead to resistance to *A. alternata*. MAPK cascades play an important role as signaling modules of a high conservation level in the response to abiotic and biotic stress and activate downstream defense-related genes (Colcombet and Hirt 2008). The MEKK1-MKK1/2-MPK4 signaling cascade in *Arabidopsis thaliana* affects both plant defense responses and the acquisition of basal resistance (Su *et al.* 2013). Our RNA-seq data revealed that the genes encoding the downstream targets of the MAPK cascades were up-regulated, which may be help pear activate the innate immune system in response to pathogen infection to produce a related immune response. Pathogens usually secrete pathogen effectors to inhibit FLS2 recognition of flg22, thus enhancing the colonization and proliferation of pathogens by overcoming PTI (Crabill *et al.* 2010). At this time, in order to resist pathogen infection, plants will further induce ETI to trigger the HR response by recognizing viral effectors through specific disease-resistance proteins (Guo *et al.* 2009). In the present study, the genes encoding RIN4, RPM1, RPS2 and EDS1 were all up-regulated. Previous studies have reported that the effectors AvrRpm1 and AvrB secreted by *Agrobacterium tumefaciens* during infection of *Nicotiana benthamiana* plants can phosphorylate the RIN4 protein to relieve its negative regulation of the disease resistance (R) protein RPM1, which limits the occurrence of disease. AvrRpt2 abolishes the inhibition of RPS2 by RIN4 by removing the physical connection between RIN4 and RPS2, ultimately triggering an R protein-mediated HR

response (Axtell and Staskawicz 2003). Therefore, the upregulation of genes encoding RPM1 and RPS2 may help to inhibit pathogen infection and activate the ETI immune system. EDS1 is a positive regulator of ETI and the up-regulated expression of EDS1-encoding genes also activates programmed cell death (Bhattacharjee *et al.* 2011). Based on these results, we can infer that PTI and ETI play roles in resisting pathogen infection during the infection and colonization of pears by *A. alternata*.

Transcription factors (TF) are widely involved in plant responses to biotic stresses and regulate the expression of defense-related genes at the transcriptional level (Sun *et al.* 2013). In our RNA-seq data, WRKY TF family genes comprised the largest group and most of the *WRKY* genes were up-regulated. Previous studies have shown that *WRKY* family genes act as positive regulators in both the Arabidopsis response to *Pectobacterium carotovorum* ssp. *carotovorum* infection and in the response to *A. alternata* infection in apple leaves (Zhu *et al.* 2017). These results indicate that up-regulated expression of *WRKY* family genes in pear may play an important role in the response to *A. alternata* infection.

MYB family genes are mainly involved in various functions such as anthocyanin biosynthesis, morphogenesis and abiotic stress responses, among others (Wang *et al.* 2004). Zhang *et al.* (2020) found that *MYB* family genes in wheat can mediate host resistance to the fungal pathogen *Bipolaris sorokiniana* by regulating the SA signaling pathway and defense-related genes. Zhu *et al.* (2017) reported that *MYB* family genes might play a regulatory role in the responses of the 'Starking Delicious' pear cultivar to *A. alternata* attack. A total of 29 pear *MYB* genes were found to be either up- or down- regulated in response to *A. alternata* infection in this experiment (Fig. 7), which suggests that these genes might also play a regulatory role in the response of Korla pear fruits to *A. alternata* infection. However, further research is needed to confirm this hypothesis.

Heat shock transcription factors (Hsfs) participate in the response to biotic and abiotic stresses by regulating the expression of heat shock-related genes (Yu *et al.* 2019). A total of six pear *Hsf* genes were either up- or down-regulated by *A. alternata* infection in this experiment (Fig. 7). Interestingly, we found that one of the down-regulated DEGs, LOC103937244, encodes a protein that is highly similar to *AtHsfB2b* from Arabidopsis. Kumar *et al.* (2009) reported that knockout of *AtHsfB2b* in Arabidopsis can significantly improve resistance to the necrotrophic fungal pathogen *A. brassicicola*. We therefore speculate that the down-regulation of this gene in pear can improve disease resistance. The other four *Hsf* genes (LOC103962963, LOC103960544, LOC103960440, LOC103960090) also showed down-regulated expression, suggesting that the defense signal transduction pathway mediated by *Hsf* TFs may be compromised in Korla fragrant pear, leading to *A. alternata* infection.

Ethylene-responsive element binding factors (ERFs) are one family of TFs that are found only in plants (Cao *et al.* 2018). Yang *et al.* (2005) reported that the *ERF4* and *ERF12* genes in Arabidopsis encode transcriptional repressors that can modulate ethylene and abscisic acid responses. In this study, the genes encoding *ERF4* (LOC103944178), *ERF12* (LOC103944179) and *ERF17* (LOC103944180) were significantly up-regulated (Fig. 7). Up-regulation of these genes negatively regulates ethylene and abscisic acid reactions, which may be related to the down-regulation of most genes in the abscisic acid and ethylene pathways (Fig. 5).

In addition, our results show that some other transcription factor family genes (*bHLH* and *ARF*) were either up- or down- regulated by *A. alternata* infection (Fig. 7). However, various studies have shown that *bHLH* and *ARF* TFs play key roles in plant growth, development and stress tolerance, but are not stronly correlated with disease resistance (Zhang *et al.* 2020). Therefore, we will not give more details on these TF families here.

The up-regulation of defense-related protein genes has been found in a variety of plants and it is an inducible part of the plant's self-defense mechanisms (Jwa *et al.* 2006). Expression of genes that encode members of the PR-1 protein family was detected in pears and most of them were found to be regulated in *A. alternata*-infected fruits. The PR-1 protein was first detected in tobacco plants infected with *Tobacco mosaic virus* and is the main PR protein induced by pathogen infection and SA (Loon and Kammen *et al.* 1970). PR-1 homologues are also found in wheat, corn and tomato plants infected by pathogens and elevated PR-1 protein levels in host plants also increase resistance to pathogens (Niderman *et al.* 1995). The genes encoding PR-1 proteins were found to be up-regulated in our experimental results and this may play a positive role in disease control. It is worth noting that up-regulation of the PR-1 protein gene is induced by the up-regulation of SA signaling pathway genes, suggesting that SA can increase resistance to necrotrophic fungi infection by inducing PR-1 proteins.

Moreover, chitinase degrades the fungal pathogen cell wall by hydrolyzing the β-1,4-glucosidic bonds between chitin N-acetylglucosamine monomers to further inhibit the infection (Okongo *et al.* 2019). It has been reported that chitinase can improve resistance to ear rot fungi in corn and resistance to the red rot pathogen (*Colletotrichum falcatum* Went) in barley and can inhibit fungal spore germination and mycelium growth (Dowd *et al.* 2018). In this study, we found that expression of 10 chitinase-encoding genes showed an up-regulation trend over the course of infection, indicating that chitinase accumulates in the pear to cope with *A. alternata* infection.

Thaumatin-like proteins (TLPs) are widely distributed in many organisms such as plants, fungi and insects (Meng *et al.* 2017). Previous studies have shown that TLPs have significant antifungal activity (Misra *et al.* 2016) and mainly work in two ways: (1) TLPs are directly inserted into the

fungal plasma membrane to form perforations, thereby destroying membrane permeability; (2) TLPs can cause enzymatic hydrolysis of β-1,3-glucan, a major part of the fungal cell wall. We identified 10 TLP-encoding genes, of which seven were up-regulated following *A. alternata* infection, with *TLP1* (LOC103935639) and *TLP1a* (LOC103962570, LOC103967809) showing significant increases in mRNA levels. These results indicate that *A. alternata* infection causes the up-regulated expression of genes encoding TLPs to resist pathogen infection in pear. Furthermore, heat shock proteins (HSPs), which act as molecular chaperones, can repair and remove the misfolded proteins produced by external factors such as plant stress, thereby reconstructing cellular protein homeostasis (Wang *et al.* 2004). Previous studies have shown that Arabidopsis infection by four pathogenic bacteria also caused the accumulation of HSP83, HSP70, HSP23.6, HSP17.6 A, and HSP17.4 (Whitham *et al.* 2003). In our experimental results, the genes encoding HSPs showed a down-regulated pattern of expression throughout the infection phase, which is consistent with results showing the down-regulation of genes encoding Hsfs transcription factors. It shows that *A. alternate*, in the process of infecting pear fruits, inhibits the expression of related transcription factors, thereby disrupting normal protein homeostasis and inhibiting the expression of defense responses. Down-regulation of the genes encoding HSP70 and HSP83 may be one of the important pathogenic mechanisms during the infection of Fragrant Pear fruits by *A. alternata*. Based on these results, we were able to draw a feasible molecular network that can explain the defense response in pear to *A. alternata* infection (Fig. 10). First of all, when pears are inoculated with *A. alternata*, the pathogen will destroy the integrity of the cell wall and a series of defense responses such as plant hormone signaling pathways (SA and JA, for example) and plant-pathogen interactions (PTI, ETI) are activated. Subsequently, TFs (WRKY, MYB, ERF, Hsfs) trigger the host responses to *A. alternata* infection by activating or inhibiting the expression of downstream genes including *CHT*, *HSP* and *PR-1*. Briefly, pears undergo specific changes in defense-related gene expression through molecular networks after infection with *A. alternata* and then produce related defense response.

Conclusion

In conclusion, in this study, a total of 25,877 DEGs were detected, and results showed that the DEGs take part in plant pathogen interactions and plant hormone signaling pathways, and defense-related proteins were up-regulated, suggesting a positive role for these genes in the pear-*A. alternata* interaction. Moreover, expression of the DEGs involved in cutin and wax biosynthesis was down-regulated, as are DEGs that encode TFs (WRKY, MYB, ERF, Hsfs) and HSP70 and HSP83, following infection of pear fruits by *A. alternata*, which may result in the appearance of blackhead disease symptoms. This study explored the defense mechanism and pathogenesis of pears in response to *A. alternata* infection and our results are expected to provide support for the development of new storage and preservation technologies for pears.

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Author Contributions

Hui Ouyang and Guogang Chen conceived and designed the experiments; Hui Ouyang and Tongrui Sun performed the experiments; Hui Ouyang, Tongrui Sun, Minrui Guo, Weida Zhang analyzed the data; Ying Jiang contributed reagents; Fund acquisition from Shaobo Cheng and Guogang Chen.

Conflict of Interest

There are no conflicts to declare.

Data Availability

The data presented in this study are available on request from the corresponding author.

Ethics Approval

There are no researches conducted on animals or humans.

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