Transcriptomics Analysis and Candidate Genes Associated With Xinjiang Jujubes in Response to *Alternaria Alternata* Infection

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Abstract

Background

Black spot disease, caused by *Alternaria alternata*, is one of the most destructive diseases of jujube worldwide. To better understand the resistance mechanisms of jujube to *A. alternata* infection to be able to improve disease control and resistance breeding. Two different cultivars, *Zizyphus jujuba* Mill. var. Jun jujube (susceptible) and *Zizyphus jujuba* Mill. var. Hui jujube (resistant), were tested.

Results

In this study, we identified 2235 differentially expressed genes (DEGs) in the disease-resistant cultivar and 4958 in the susceptible cultivar. To better understand these DEGs, the datasets were analyzed using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genome (KEGG) database. Most of them were associated with plant phytohormone synthesis and signal transduction, flavonoid synthesis, and glutathione metabolism. The expression of 6 DEGs associated with disease resistance were detected by real time-quantitative polymerase chain reaction (RT-qPCR), consistent with the results of Illumina transcriptome sequencing. Moreover, the expression level of the six DEGs differently in Jun jujube and Hui jujube, verified they are defense response factors.

Conclusions

The present study identified several candidate resistance genes and signal transduction pathways that may contribute to black spot disease resistance in jujube, which will assist the investigation of resistance mechanisms in the response of jujube to *A. alternata* infection.

Background

Black spot disease is a common disease in most parts of the world where jujubes are grown, mainly caused by *Alternaria alternata*, which is a biotrophic oomycete with a narrow host range [1, 2]. As Xinjiang has one of the highest production level of jujube in the world, black spot disease is a major threat that has caused great economic in the area [3]. To protect against this pathogen and produce healthy fruit, jujube yards require frequent fungicide applications, a practice that is not only costly for the grower but also contributes to environmental pollution and may negatively impact human health [4]. However, both the rate of occurrence and severity of black spot disease vary between jujube cultivars. For example, the Hui jujube and Hami jujube varieties are resistant to *A. alternata*, while the Jun jujube and Dong jujube strains are highly susceptible to infection [5, 6]. Thus, extensive breeding research has been carried out to understand the underlying genetics and mechanism of resistance to this disease so that new, less-susceptible cultivars can be produced. Nevertheless, little progress has been made, mainly because of the limited understanding of the genetic response to the pathogen.

Previous studies on black spot disease in jujube have mainly focused on the identification of the pathogen [7-9], the environmental antibiotics for controlling *A. alternata* [10, 11], and the detection of *Alternaria* toxins [12]. Little studies have evaluated disease resistance in jujube germplasm resources. The only source of resistance described to date is governed by a major gene with a stable heritance; the molecular mechanisms of the plant-pathogen interactions, however, remain uncertain.

Transcriptome analysis makes it possible to obtain comprehensive transcription sequence information of a specific tissue or organ in a specific state without the need for detailed genome information [13]. It has also been used to study genes and genetic mechanisms of plant-pathogen interactions, in a variety of systems including cucumber-*Alternaria cucumerina* [14], grape-*Plasmopara viticola* [15], apple-*A. alternata* apple pathotype [16], and others. To explore the mechanisms of the response of jujube fruit to pathogens and establish the genetic basis for the development of resistant varieties, many RNA-seq studies have been conducted on the interaction between jujube and pathogens or other stresses, including jujube witches' broom caused by *Candidatus Phytoplasma ziziphi* [17], cold stress [18] and salt stress [19].

It is important to understand the resistance mechanisms of jujube to *A. alternata* infection to be able to improve disease control and resistance breeding. In this study, we conducted a RNA-seq to investigate the responses of two jujube varieties, to inoculation with *A. alternata*, a resistant type (Hui jujube) and a susceptible one (Jun jujube). The findings will assist the investigation of resistance mechanisms in the response of jujube to *A. alternata* infection.

Results

Overview of all RNA-Seq data of the Jujube

A total of 198 Gb raw data were generated from 30 samples using the Illumina HiSeq™2500 platform. The raw data were uploaded to NCBI (reviewer link: <https://www.ncbi.nlm.nih.gov/sra/PRJNA772684>). Ultimately, the transcripts generated at least 6.6 Gb raw reads from each sample, after filtering (Q 20% > 97.35%), at least 6.43 Gb clean reads acquired from each sample. About 90.89% of the clean reads were mapped to the *Zizyphus jujuba* reference genome (GCF_000826755.1_ZizJuj_1.1_genomic.fna). Of the mapped reads, 82.51% were mapped uniquely (Additional file 1: Table S1).

The expression of the same gene in different splicing forms may cause completely different biological effects. FPKM density distribution is used to inspect gene expression patterns. Fig. 1 showed that the majority of genes in all the samples were moderate expressed, while only a small part were low and high expressed. The results indicated that the gene expression level of each sample was moderate and could be used for subsequent analysis.

To display the amount of gene data under different FPKM values, the number of genes with FPKM values between 0–1000 and FPKM > 1000 and their proportion of the total genes were quantified. After *A. alternata* infection for 1–4 days, the distribution of gene expression and number of FPKM > 1 were as follows: in Jun jujube 41.58% and 12477, 45.52% and 13659, 42.84% and 12858, and 43.08% and 12930 for days 1–4, respectively. The expression and gene

number were the most on the day 2 (as shown in Additional file 3: Fig. S1). The values for Hui jujube were 43.38% and 13017, 43.19% and 12960, 42.03% and 12613, and 46.17% and 13856, respectively, with the highest value on the day 4 (as shown in Additional file 4: Fig.S2).

Correlation among gene expression levels between samples is an important indicator of the reliability of experiments and the reasonableness of sample selection. Pearson correlation analysis was conducted for three biological replicates in each sample according to the gene expression level of each sample. The closer the Pearson correlation coefficient R of the two samples is to 1, the stronger the correlation between the two samples is. As can be seen in Fig. 2, the correlation coefficient R of Jun jujube and Hui jujube samples was 0.851–1, which is highly correlated, meeting the requirements of subsequent DEGs analysis.

Saturation analysis and gene coverage evaluation of the tested samples were used to further evaluate the robustness of our sample. Gene expression levels did not significantly change with an increase in the number of samples considered, which indicated that the saturation of the tested samples was of high overall quality and could cover a large area and satisfy more than 95% of the expressed genes. The gene coverage curve reflects whether the sequence is evenly distributed on the gene. When the data curve is smooth and there is no obvious peak value at the left or right ends, it indicates that the sequencing result is unbiased and relatively uniform and reliable, which can be used to analyze subsequent test data. As can be seen in Fig. 3 and 4, the overall quality of sequencing saturation and gene coverage of Jun jujube and Hui jujube after 4 days of infection by *A. alternaria* were relatively high, covering the majority of expressed genes with uniform results and reliable data.

DEG responses to *A. alternata*

In this study, 24 comparison groups, relating to the samples of Hui jujube and Jun jujube grown at 1, 2, 3 and 4 day after inoculation with *A. alternata* 224T-1, were constructed as summarized in Table 1. Significantly differentially expressed transcripts were noted among Hui jujube samples at different days after inoculation (Fig. 5), with 602 DEGs upregulated and 187 DEGs downregulated in the H-CK-vs-H-T1, and 619 and 671, respectively, in H-T1-vs-H-T2. For Jun jujube, the values were 1026 and 307 in J-CK-vs-J-T2, and 1226 and 695 in J-T1-vs-J-T2. There were approximately two-fold more DEGs upregulated DEGs associated with Jun jujube than Hui jujube. In same day 2 comparison, 2944 DEGs were upregulated and 2677 were downregulated in H-T2-vs-J-T2. These results indicate that inoculation stress had a greater effect on Jun jujube than on Hui jujube, in other words, the response to *A. alternata* showed a declining trend in Hui, however, remained basically stable in Jun. Moreover, a Venn diagram analysis (Fig. 6) revealed that 71 DEGs were expressed on all days in Jun jujube; Hui jujube had 12 such DEGs.

Table 1 Comparison groups constructed in the analysis of DEG responses to *A. alternata*

No.	Comparison groups	No.	Comparison groups
1	H-CK-vs-H-T1	13	J-CK-vs-J-T1
2	H-CK-vs-H-T2	14	J-CK-vs-J-T2
3	H-CK-vs-H-T3	15	J-CK-vs-J-T3
4	H-CK-vs-H-T4	16	J-CK-vs-J-T4
5	H-T1-vs-H-T2	17	J-T1-vs-J-T2
6	H-T1-vs-H-T3	18	J-T1-vs-J-T3
7	H-T1-vs-H-T4	19	J-T1-vs-J-T4
8	H-T2-vs-H-T3	20	J-T2-vs-J-T3
9	H-T2-vs-H-T4	21	J-T2-vs-J-T4
10	H-T3-vs-H-T4	22	J-T3-vs-J-T4
11	H-T1-vs-J-T1	23	H-T3-vs-J-T3
12	H-T2-vs-J-T2	24	H-T4-vs-J-T4

Go functional analysis of DEGs

GO functional analysis was performed on eight groups (H-CK-vs-H-T1, H-CK-vs-H-T2, H-CK-vs-H-T3, H-CK-vs-H-T4, J-CK-vs-J-T1, J-CK-vs-J-T2, J-CK-vs-J-T3, and J-CK-vs-J-T4) to explore the functions of the DEGs that responded to *A. alternata* 224T-1 infection (Additional file 5: Fig. S3; Additional file 6: Fig. S4). DEGs with uncertain functional classification were grouped into one of three categories: cellular components (CC), molecular function (MF), and biological processes (BP). Among them, the differentially expressed BP-GO term genes of Hui jujube were mainly concentrated in localization, single biological process, response to stimulus, metabolic process, and cellular process; Those of Jun jujube mainly included metabolic processes, cellular processes, localization, single-organism processes, response to stimuli, and biological regulation.

MF DEGs were mainly concentrated in binding, transporter activity, catalytic activity, and structural molecule activity for Hui jujube and catalytic activity, binding, transporter activity, electron carrier, and signal transducer activity for Jun jujube. After infection, DEGs were significantly enriched in cell parts, membrane parts, organelles, membranes, and organelle parts in both cultivars.

Pathway analysis of DEGs

Next, the DEGs of the same eight comparisons were compared against the KEGG pathway database, and pathways with P value < 0.05 were screened out (Table 2). There were 20 pathways of 34 DEGs enriched in Jun jujube. These related to the response to pathogen infection, including plant hormone signal transduction, glutathione metabolism, flavonoid biosynthesis, fatty acid metabolism, and alpha-linolenic acid metabolism. For Hui jujube, 21 DEGs were screened out with 13 pathways including plant hormone signal transduction, pentose phosphate pathway, and diterpenoid biosynthesis.

Table 2 Metabolic pathways in Jun jujube and Hui jujube

Sample name	Pathway ID	Pathway	DEG number	P-value
J_CK_vs_J_T1	zju04141	Protein processing in endoplasmic reticulum	40	3.20765E-20
	zju00592	Alpha-Linolenic acid metabolism	9	7.70762E-05
	zju00591	Linoleic acid metabolism	5	0.000294098
	zju00905	Brassinosteroid biosynthesis	5	0.004119132
J_CK_vs_J_T2	zju00195	Photosynthesis	8	0.000362064
	zju00592	Alpha-Linolenic acid metabolism	9	0.000491787
	zju00360	Phenylalanine metabolism	9	0.001054213
	zju04626	Plant-pathogen interaction	17	0.004089041
J_CK_vs_J_T3	zju00943	Isoflavonoid biosynthesis	6	1.94658E-07
	zju00620	Pyruvate metabolism	13	0.000180635
	zju04626	Plant-pathogen interaction	21	0.000191438
	zju00020	Citrate cycle (TCA cycle)	9	0.000361880
	zju00250	Alanine, aspartate and glutamate metabolism	9	0.000646099
	zju00591	Linoleic acid metabolism	5	0.001179480
	zju00061	Fatty acid biosynthesis	8	0.001701428
	zju00511	Other glycan degradation	5	0.001917700
	zju00780	Biotin metabolism	5	0.001917700
J_CK_vs_J_T4	zju00592	Alpha-Linolenic acid metabolism	12	8.06453E-06
	zju00010	Glycolysis/Gluconeogenesis	16	0.000753532
	zju04626	Plant-pathogen interaction	19	0.002296723
	zju00061	Fatty acid biosynthesis	8	0.002321533
	zju00620	Pyruvate metabolism	11	0.003330230
	zju00230	Purine metabolism	11	0.004286921
	zju00943	Isoflavonoid biosynthesis	3	0.004763506
	zju00591	Linoleic acid metabolism	4	0.010432218
	zju00902	Monoterpenoid biosynthesis	9	0.011109653
	zju00261	Monobactam biosynthesis	3	0.012638292
	zju00511	Other glycan degradation	4	0.014964785
	zju00780	Biotin metabolism	4	0.014964785
	zju04141	Protein processing in endoplasmic reticulum	17	0.017020575
	zju00920	Sulfur metabolism	6	0.017508284
	zju00905	Brassinosteroid biosynthesis	5	0.017553936
	zju00450	Selenocompound metabolism	4	0.023802163
	zju00195	Photosynthesis	5	0.048821916
H_CK_vs_H_T1	zju00941	Flavonoid biosynthesis	6	0.000873849
	zju00052	Galactose metabolism	3	0.003412325
	zju00740	Riboflavin metabolism	2	0.034400936
	zju00780	Biotin metabolism	3	0.037935319
	zju04712	Circadian rhythm-plant	2	0.040701275
	zju00290	Valine, leucine and isoleucine biosynthesis	11	8.67806E-07
H_CK_vs_H_T2	zju04016	MAPK signaling pathway-plant	3	0.021510839
	zju00945	Stilbenoid, diarylheptanoid and gingerol	8	0.022638130
	zju00520	Amino sugar and nucleotide sugar metabolism	4	0.025028043

	zju00941	Flavonoid biosynthesis	6	0.032101765
	zju00270	Cysteine and methionine metabolism	9	0.040083482
	zju00940	Phenylpropanoid biosynthesis	4	0.043787242
	zju00360	Phenylalanine metabolism	9	6.10142E-09
H_CK_vs_H_T3	zju00941	Flavonoid biosynthesis	2	0.003487582
	zju00943	Isoflavonoid biosynthesis	12	4.59014E-12
H_CK_vs_H_T4	zju00941	Flavonoid biosynthesis	6	0.000873849
	zju00052	Galactose metabolism	3	0.003412325
	zju00740	Riboflavin metabolism	2	0.034400936
	zju00780	Biotin metabolism	3	0.037935319
	zju04712	Circadian rhythm—plant	2	0.040701275
	zju00290	Valine, leucine and isoleucine biosynthesis	6	0.000873849

DEGs involved in phytohormone signaling. As shown in Fig. 7, after *A. altrenata* infection, the expression of three genes in the auxin (AUX /IAA)—mediated signaling pathway were changed and two of them regulating AUX were upregulated 5.1-fold, one gene regulating ARF was downregulated by 0.4-fold; one of the three genes regulating SAUR was up-regulated 13.9-fold; and the other two were downregulated 0.4-fold. The expression of two genes in the AB-mediated signaling pathway was changed, among them the genes regulating SNRK2 were upregulated 2.4-fold, and the genes regulating ARF were down-regulated 0.4-fold. The expression of several genes in the ethylene signaling pathway was changed, among which the regulatory ERF (Ethylene response factor) gene was upregulated by 7.9 and 7.0-fold, respectively. The expression of related genes in BRs signaling pathway was changed, in which BAK1 gene was upregulated 2.7-fold and Bri1 gene was upregulated 2.0-fold. The expression of several genes of BRS pathway in Jun jujube was changed, which suggested that BRS signaling pathway was involved in the response process of Jun jujube to *A. altrenata*. JAZ related genes in JA signaling pathway were up-regulated by 5.5-fold, suggesting that JA signaling pathway was involved in *A. altrenata* response. TGA gene was upregulated by 15.8-fold, and involved in downstream regulation of NPR1 expression, suggesting that SA signaling pathway was involved in *A. altrenata* response.

Analysis of flavonoid metabolism pathway and related genes. As Shown in Fig. 8, seven genes related to flavonoid biosynthesis were expressed and involved in regulation, including chalcone synthase (CHS, EC2.3.1.74) upregulated by 29.1, 18.6 and 3.0-fold, respectively. The dihydroflavonol reductase (DFR, EC1.1.219) was increased by 23.9 and 19.2-fold, respectively. The flavonoid 3'-hydroxylase (F3H, EC1.14.11.9) was increased 13.7-fold; Gene CYP73A (EC1.14.14.91) was upregulated by 47.3, 7.9 and 2.3-fold, and gene CYP75B1 (EC1.14.14.82) was upregulated by 3.8-fold. Anthocyanidin synthase (ANS, EC1.14.20.4) was increased by 65.5 and 5.8-fold, respectively. Anthocyanidin reductase (ANR, EC1.3.1.77) was increased by 5.0-fold. Leucoanthocyanidin reductase (LAR, EC1.17.1.3) gene was increased by 30.5-fold.

Metabolic pathways and related genes of glutathione. As shown in Fig. 9, the expression of genes related to glutathione metabolism changed, including those regulating GGT (EC2.3.2.2) by 3.5-fold and GGT5 (EC2.3.2.2) by 3.5-fold. Three genes regulating glutathione transferase (GST, EC2.5.1.18) were upregulated by 5.0, 2.2 and 0.4-fold, respectively. One gene regulating ribonucleoside-diphosphate reductase large subunit (RRM2, EC1.17.4.1) was down-regulated by 0.3-fold. One gene regulating γ -glutamylcysteine synthetase (γ -ECS, EC6.3.2.2) was upregulated 3.5-fold and EC4.1.1.17 was upregulated 7.5-fold.

Validation of RNA-seq data by qRT-PCR

To further verify the results of transcriptome sequencing and explore the expression of DEGs in each cultivar, six genes related to disease resistance and showing the largest difference between cultivars were selected from the metabolic pathways of plant hormones, flavonoid biosynthesis and glutathione: *GMP synthase*, *expansin-A1*, *glucose-6-phosphate/phosphate translocator 2*, *amino acid permease*, *protein PTR protein family 5.4*, and *sulfate transporter 3.4*.

qRT-PCR was used to verify the DEGs recognized by RNA-seq; the trends in the gene expression pattern concurred with the gene expression pattern recognized by RNA-seq (as shown in Fig. 10), so the Illumina sequencing results were considered relatively reliable.

As shown in Fig. 11, there were significant differences among the six resistance genes and the expression of *GMP synthase*, *expansin-A1* and *amino acid permease* basically increased first and then decreased under different inoculation times. Expression levels were higher in Hui jujube than in Jun jujube. The expression of *glucose 6-phosphate/phosphate translocator 2* followed the same pattern in Jun jujube and its highest level was observed on day 2, at which was 7.5-fold higher than that on day 0. *PTR FAMILY 5.4* showed the same up-the-down pattern in Jun jujube but gradually increased over time in Hui jujube. Finally, *sulfate transporter 3.4* showed the up-down pattern in Hui jujube but gradually increased in Jun jujube.

Discussion

In this study, Illumina HiSeq™2500, a high-throughput sequencing platform, was used to conduct transcriptome sequencing and functional analysis of two jujube varieties exposed to *A. altrenata* infection. The criteria for selecting DEGs was $|\text{Log}_2^{\text{fold change}}| \geq 1$, and P-value ≤ 0.5 ; ultimately, 2944 upregulated genes and 2677 downregulated genes were obtained.

Expression levels of genes involved in phytohormone synthesis and signaling transduction

Phytohormones, including salicylic acid (SA), ethylene (ET), brassinosterols (BR), abscisic acid (ABA), and jasmonic acid (JA), are critical regulators of plant–pathogen interactions. In response to stress, plants activate the expression of related defense genes by regulating the synthesis of various hormones, which is an effective way to resist disease invasion [20]. In jujube fruit, *A. altrenata* infection alters the expression of genes involved in phytohormone biosynthesis and signal transduction, including the up- or downregulation of genes related to IAA, ABA, ET, BR, JA, and SA. All of the DEGs involved in biosynthesis and signal transduction of ET, BR, JA, and SA were upregulated on the first day after treatment. In contrast, some DEGs related to IAA and ABA were downregulated on the first day. We hypothesize that *A. altrenata* targets the host's phytohormone system during its primary invasion stage, and that the downregulated DEGs may be host- protective responses.

ET and JA are key signal molecules that induce systemic resistance, while SA activates the expression of WRKY, the TGA family, PRs, and other resistance genes through its interaction with the downstream positive regulator NPR1 enhancing plant resistance to trophic pathogens [21]. In this study, the genes encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-formic acid oxidase (ACO), key enzymes in ET biosynthesis, were significantly upregulated, while the ET receptor gene ETR, a negative regulator of ethylene reaction, was significantly downregulated in the ET signal transduction pathway. The downstream ethylene response factor gene ERF 1/2 was significantly upregulated. ERFs can regulate the expression of PR1, PR2, PR3, PR5, and other PRs-coding genes through the structural element GCC-box, so as to trigger a corresponding disease resistance response [22]. Guo *et al.* (2016) [23] showed that Pti5 is a transcription regulator of the PR gene and the upregulated expression of PR5 gene was directly regulated and involved in fruit resistance; our sequencing result are consistent with those findings.

SA, a simple phenolic substance produced in plants, is an important signaling molecule in disease resistance [24], which acts by regulating various enzyme activities and inducing pathology-related proteins. Among them, nonexpressor of prgenes 1 (NPR1) is essential [25]. In our study, NPR1 transcription factor and PR1 resistance protein genes in the SA metabolism pathway were upregulated 7.51- and 4.51-fold, respectively, while phenylalanine ammonialyase encoding gene PAL was also significantly upregulated.

JA is a main hormones in this process, and the jasmonate ZIM domain (JAZ), an inhibitor of the JA pathway, is a key link in the induction of plant disease resistance by JA and its derivatives [26]. In our study, most DEGs involved in JA biosynthesis and the signal pathway were upregulated. Expression of JAZ, was consistently upregulated 5.5-fold. Shigenage *et al.* (2016) [27] reported that the JAZ protein family can bind to the positive JA response transcription factor MYC2 to inhibit the expression of JA early response genes in the JA signaling pathway.

BRs can increase plant resistance to chilling injury, freezing injury, disease, herbicides and salt injury; coordinate the relative levels of endogenous hormones in plants; stimulate enzyme activity; and affect gene expression [28]. In our study, the expression of genes involved in the BR signaling pathway was changed, where the BAK1 gene was upregulated 2.7-fold and the Bri1 gene was upregulated 2.0-fold. Both BSK1 and BAK1 are members of the Bri1 receptor-related kinase family; they interact with Bri1 to transduce plant BRS signals from extracellular to intracellular components, and play an important role in regulating the balance between plant growth and development and disease resistance [29]. The expression of several genes in the BR pathway was changed in Jun jujube fruits, suggesting that this pathway was involved in the response to *A.altrenata*.

Expression levels of genes involved in flavonoid metabolism

Secondary metabolism is derived from primary metabolism and is the result of adaptation of plants to the environment. After pathogen infection, plants produce and accumulate a large number of secondary metabolites, among which flavonoid, phenylpropane, terpenoids, and steroids are the main types [30]. Flavonoids, as important secondary metabolites, are ubiquitous in plants and have functions of regulating immunity, antioxidation and antimicrobial activity. They play an important role in signal transduction and plant resistance to external stress [31]. Our results indicate that the genes related to the activation of secondary metabolism of Jun jujube were activated after infection by *A.altrenata*: *chalcone synthase (CHS)*, *dihydroflavonol reductase (DFR)*, *flavonoid 3'-hydroxylase (F3H)*, *gene CYP73A*, *gene CYP75B1*, *anthocyanidin synthase (ANS)*, *anthocyanidin reductase (ANR)*, *leucoanthocyanidin reductase (LAR)*. This suggests that secondary metabolism, particularly flavonoid metabolism, plays an important role in disease resistance in Jun jujube.

Expression levels of genes involved in glutathione metabolism

Glutathione (GSH) is widely found in plants and plays a key role in resistance to environmental stress, maintenance of antioxidant properties, and regulation of signal transduction sensitive to redox [32]. GST, as one of the most important enzymes of GSH, plays an important and special role. Changes in GST activity are closely related to plant resistance to environmental stress [33]; enhanced expression of GST, an important marker of the plant response to stress [34]; and γ -G lutamylcysteine synthetase (γ -ECS), a key enzyme in the GSH biosynthesis pathway, that regulates the level of GSH biosynthesis [35]. In our study, three GST genes were identified in Jun jujube after *A. altrenata* infection. Previous studies have shown that GST genes play different roles in plant defense against fungal infection.

Conclusion

As black spot disease caused by *A. altrenata* infection is a common disease in jujube, it is important to understand the resistance mechanisms for improving disease control and resistance breeding. Overall, we found that most DEGs in Jun jujube showed obvious differences, mainly in phytohormones, flavonoids, and glutathione. It is speculated that the resistance of Jun jujube to pathogens can be achieved by regulating the expression of phytohormones, flavonoid biosynthesis and glutathione metabolism. The significant expression of *GMP synthase*, *expansin-A1*, *glucose-6-phosphate/phosphate translocator 2*, *amino acid permease*, *protein PTR protein family 5.4*, and *sulfate transporter 3.4* may represent a defense response.

Methods

Plant materials, *A. altrenata* culture, and inoculation method

The two jujube varieties used in this study, were *Zizyphus jujuba* Mill. var. Hui jujube and *jujuba* Mill. var. Jun jujube, which were provided by the Jujube Test Station located in the 2nd Company, the 224th Regiment, Xinjiang Production and Construction Corps. The former is resistant to *A. altrenata*, as identified via a preliminary experiment (incidence rate: 3.30%), and the latter is susceptible to it (incidence rate: 31.65%).

A. altrenata 224T-1 (obtained from Jun jujube infected with black spot and identified by Sangon Biotech (Shanghai) Co., Ltd.) was incubated on potato dextrose agar (PDA) in Petri dishes at 26°C for 7–10 days in the dark. A suspension of conidium were collected in tween-water and adjusted to 1×10^7 conidium·mL⁻¹ via dilution and electron microscope. The tested jujube fruit in white ripe stage were sprayed with a suspension of conidium (1×10^7 conidium·mL⁻¹). Control jujube fruit were sprayed with sterilized water. All the jujube fruit in treatment groups were covered with 10 mL sterilized water to maintain the relative humidity. Inoculated jujube samples, obtained 1, 2, 3, and 4 days after inoculation, were placed in liquid nitrogen and stored at -80°C for RNA extraction. Each sample contained five jujube fruits from each three jujube trees representing three biological replicates.

Total RNA extraction and quality determination

According to the Mini BEST Plant RNA Extraction Kit (TaKaRa)' handling instruction, total RNAs were extracted from 0.3 g jujube tissue. RNA purity and concentration were measured at 260/280 nm using a spectrophotometer (NanoDrop-2000, Thermo Scientific). RNA integrity was verified via agarose gel electrophoresis and examined using an Agilent 2100 Bio analyzer.

Library construction and Illumina sequencing

Methods referred to Zhu's [16] were modified as follows: samples were pooled to construct a complementary DNA (cDNA) library. Among them, poly-(A) messenger RNAs (mRNAs) were isolated from total RNA using Oligo (dT) magnetic beads and fragmented in fragmentation buffer. Then, using these short fragments as templates, random hexamer-primers were then used to synthesize first-strand cDNA, while second-strand cDNA was synthesized using buffer, dNTPs, RNaseH and DNA polymerase I. Short double-stranded cDNA fragments were purified using a TaKaRa Prime Script TM RT Reagent Kit with gDNA Eraser. Products were purified (AMPure XP system) and quantified using the Agilent high- sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The obtained libraries (~450 bp) were sequenced on the Illumina HiSeq™ 2500 platform at the Shanghai Personal Biotechnology Co., Ltd.

Illumina reads and DEG

All downstream analyses were based on high-quality clean data. These clean data were obtained using the FASTQ_Quality_Filter tool from the FASTX-toolkit by removing low-quality reads (the number of Qphred ≤ 20 bases occupy more than 50% of the total read length) and reads containing poly-N ratio ≥ 10 from raw data. Clean data were mapped onto the reference genome sequence Chinese Long V2 using HISAT 2.0.4 (the ratio of total mapped reads ≥ 70 and multiple mapped reads ≤ 10). HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. The expression levels of genes were quantified in terms of fragment per kilobase per million mapped (FPKM) using the software RNA-Seq by Expectation Maximisation (RSEM). DEGs were detected using the DESeq2 R package (1.18.0), and are illustrated herein using Venn diagrams. Genes with an adjusted P-value < 0.05 and $|\text{Log}_2^{\text{fold change}}| \geq 1$ were identified as DEGs.

Gene ontology and KEGG pathway analysis of DEGs

The GOeast package and KOBAS software were used to analyze the statistical enrichment of upregulated and downregulated DEGs in GO terms and KEGG pathways with adjusted P-values ≤ 0.05 considered significantly enriched.

RT-qPCR analysis

To prove the expression profiles of DEGs obtained through RNA-seq, 6 DEGs were chosen for analysis via RT-qPCR analysis. Gene sequences were obtained from the *Zizyphus jujuba* Genomics Database (https://www.ncbi.nlm.nih.gov/genome/15586?genome_assembly_id=219393). Gene-specific primers were designed online (<https://www.sangon.com/newPrimerDesign>) and are presented in Additional file 2: Table S2. RNA was extracted using the Mini BEST Plant RNA Extraction Kit (TaKaRa) and reverse-transcribed to cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). RT-qPCR was performed using the Taq SYBR Green qPCR Premix kit (TaKaRa). The relative expression was analyzed according to the $2^{-\Delta\Delta Ct}$ method, with actin as the internal reference gene to normalize the data.

Abbreviations

DEGs: Differentially Expressed Genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genome; RT-qPCR: Real Time-quantitative Polymerase Chain Reaction; CC: Cellular Components; MF: Molecular Function; BP: Biological Processes; ERF: Ethylene Response Factor; CHS: Chalcone Synthase; DFR: Dihydroflavonol Reductase; F3H: Flavonoid 3'-Hydroxylase; ANS: Anthocyanidin Synthase; ANR: Anthocyanidin Reductase; LAR: Leucoanthocyanidin Reductase; GST: Glutathione S-transferase; γ -ECS: γ -glutamylcysteine synthetase; SA: Salicylic acid; ET: Ethylene; BR: Brassinosterols; ABA: Abscisic acid; JA: Jasmonic acid; IAA: Indole-3-acetic acid; ACS: 1-aminocyclopropane-1-carboxylic acid synthase; ACO: 1-aminocyclopropane-1-formic acid oxidase; NPR1: Nonexpressor of prgenes 1; JAZ: Jasmonate ZIM domain; CHS: chalcone synthase; DFR: Dihydroflavonol reductase; LAR: Leucoanthocyanidin reductase; GSH: Glutathione; PDA: Potato Dextrose Agar; cDNA: complementary DNA; FPKM: Fragment per kilobase per million mapped; RSEM: RNA-Seq by Expectation Maximisation.

Declarations

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Authors' contributions

CW was the recipient of funds; YF, RZ, AW, and CW conceived the experiment. RZ, XL, and YM undertook experiments and data analysis. FL, WH and YW collected samples. YF, RZ, XL, and CW prepared the manuscript. All of the authors reviewed and accepted the final version of the manuscript.

The authors read and approved the final manuscript.

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Availability of data and materials

The RNA-Seq data are available in the Sequence Read Archive (SRA) repository of National Center for Biotechnology Information (NCBI) with the accession numbers PRJNA772684.

Ethics approval and consent to participate

The current study complies with relevant institutional, national, and international guidelines and legislation for experimental research and field studies on plants (either cultivated or wild), including the collection of plant material.

Consent for publication

Not applicable

Competing interest

The authors declare no competing interests.

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Figures

Figure 1

FPKM density distribution

(The x axis represents the gene distribution density corresponding to the expression level; the horizontal lines in the middle of each box are the median, upper and lower edges of the box are 75%, upper and lower limits are 90%; external shape is the kernel density estimate. J represents Jun jujube; H represents Hui jujube; CK represents clear water control group; T represents treatment group; TX (X=1, 2, 3, 4) represents X day after infection treatment.)

Figure 3

Saturation analysis of Hui (A) and Jun (B) infection with *A. alternaria* after 4 days

(Genes are divided into four groups according to expression level. Q1: genes with expression levels in the bottom 25%; Q2: genes with expression levels in the bottom 25–50%; Q3: genes with expression levels in the top 25–50% of genes; Q4: genes with the highest expression level in the top 25%.)

Figure 4

Gene coverage of Hui (A) and Jun (B) infection with *A. alternaria* after 4 days

Figure 5

Statistics of DEGs between samples

(Horizontal axis represent each comparison; red indicates upregulated genes, blue indicates downregulated genes.)

Figure 6

Common differential genes among Jun jujube samples (A) and Hui jujube samples (B)

Figure 7

Phytohormones signaling pathway involved in differential genes in Jun jujube

Figure 8

Flavonoid biosynthesis pathway involved in DEGs in Jun jujube

Figure 9

Glutathione metabolic pathway involved in DEGs in Jun jujube

Figure 10

Verification of the DEGs via qRT-PCR in Hui (A) and Jun (B) jujube

(P1: GMP synthase; P2: expansin-A1; P3: Glucose-6-phosphate/phosphate translocator 2; P4: Amino acid permease; P5: PTR FAMILY 5.4; P6: Sulfate transporter 3.4)

Figure 11

qRT-PCR analysis of disease-related genes at different times after infection with *A. alternaria*

(P1: *GMP synthase*; P2: *expansin-A1*; P3: *glucose-6-phosphate/phosphate translocator 2*; P4: *amino acid permease*; P5: *PTR FAMILY 5.4*; P6: *sulfate transporter 3.4*)

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