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Transcriptome Sequencing and Analysis of Genes Related to Disease Resistance in *Pinus thunbergii*

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Abstract: *Pinus thunbergii* (*P. thunbergii*) is a gymnosperm with important economic and ecological value. In order to investigate the diagnosis and defense mechanism of *P. thunbergii* against *Bursaphelenchus xylophilus* (the pinewood nematode, PWN), the needles of *P. thunbergii* seedlings on the fifth day after being infected by PWN were taken as samples for transcriptome sequencing analysis. Compared with the control group, 647 genes were differentially expressed in the treatment group, of which 277 genes were upregulated and 370 genes were downregulated. Enrichment analysis showed that most of these differentially expressed genes were abundant in the biosynthesis of secondary metabolites, pathogen interaction and hormone signal transduction. In addition, among the differential genes, *NBS-LRR* genes, thiamine-metabolizing enzymes, phenylalanine ammonia lyase and acetaldehyde dehydrogenase were screened and analyzed. The analysis of the response of *P. thunbergii* to PWN stress and its disease resistance genes lays a foundation for the breeding of disease-resistant *P. thunbergii* in the future.

Keywords: *Pinus thunbergii*; transcriptome; resistance; *Bursaphelenchus xylophilus*; gene

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1. Introduction

Black pine (*Pinus thunbergii* Parl., *P. thunbergii*) is a dominant coastal shelterbelt plant in Shandong, China [1], with high tolerance to conditions in harsh environments such as desertification and high salinity. Pine wilt disease (PWD) is a destructive disease caused by *Bursahelenchus xylophilus* (pinewood nematode, PWN) infection [2,3]. It mostly spreads by *Monochamus alternatus* [4,5] in summer. The PWN was first discovered in 1934 [3] and was subsequently introduced to the Chinese mainland [6], Taiwan [7] and South Korea [8] in the 20th century, causing major damage in a short period of time. Therefore, PWD is one of the main risks of black pine production.

Once infected by PWN, parenchyma cells in pine trees produce a large number of terpenoids, and the ability to resist and avoid nematodes is improved [9]. However, the evaporation of terpenoids can induce xylem cavitation, resulting in the death of pine trees [10]. At the same time, this is accompanied by other symptoms, such as the increase in total phenols and total flavonoids [11], and the enrichment of early plant disease resistance hormones including salicylic acid and methyl jasmonate [12], which contribute to the defense of pine against PWD.

When woody plants are invaded by disease, gene expression also changes. Daniel et al. reported that the expression of genes related to catalysis, metabolism, hydrolase and transferase increased significantly at 24 h and 48 h after *P. yunnanensis* was infected by PWN [13]. There were different expression levels of genes related to biological processes, including phenylpropanide biosynthesis, flavonoid biosynthesis, and redox and plant-type allergic reactions in *P. koraiensis* infected with PWN [14]. Li et al. reported the differentially expressed genes (DEGs) related to the metabolic pathway, secondary metabolites, phenylpropanoid biosynthesis and carbon fixation during photosynthesis in tea leaves [15]. Liu et al. provided information on DEGS related to hormone homeostasis, photosynthesis

and signal transduction in stress-tolerant and stress-sensitive wheat [16]. At present, the genome sequencing of black pine has not been reported, the genetic background is not clear, and the research on molecular disease resistance is limited. Studying the disease resistance of black pine from the perspective of molecular biology is of great significance to cultivate black pine seedlings that are resistant to PWN.

In our study, transcriptome sequencing technology was used to identify the DEGs in needles of black pine seedlings after 5 days of PWN infection, and the genes related to PWD resistance were screened and analyzed, which is of great scientific significance to further study the function and related biological processes of disease resistance genes in black pine.

2. Materials and Methods

2.1. Materials

The tested materials were two-year-old black pine seedlings (wild-type, Japanese black pine, bought from Rizhao, China), potted in the greenhouse of Qingdao University, with a stem height of approximately 50 cm. PWN were isolated from susceptible withered branches of black pine (collected from Shandong, China) by the Behrman funnel method [17], inoculated on Potato Dextrose Agar (PDA) medium full of Botrytis cinerea and cultured in the dark at 25 °C. A PWN suspension (2500 nematodes/100 μL) was prepared, and 15 black pine seedlings were infected by artificial skin grafting [18] as the treatment group (BL); another 15 black pine seedlings were treated with sterile water as the control (CK). On the first day, third day, fifth day and seventh day after infection, one plant was randomly selected from the CK and the BL, respectively, for the isolation and identification of PWN. The black pine was cut into multiple segments, including 10 cm above and below the infection site, stem tip, lateral branches and needles, and the movement range of PWN was identified. At the same time, the needles were taken on the first, third, fifth, seventh, tenth and fifteenth day after infection. After quick freezing with liquid nitrogen, they were stored in the refrigerator at −80 °C to await further experiments. The phenotype of the black pine seedlings was observed continuously for 3 months.

2.2. RNA Extraction, Library Construction and Sequencing

According to the manufacturer's operating instructions, the total RNA of the needles of the control group (CK) and the treatment group (BL) was extracted using a Trizol Kit (Invitgen, Carlsad, CA, USA). Random primers were used to reverse transcribe RNA into the first-strand cDNA, and then the second-strand cDNA was synthesized. The cDNA production was purified using a QiaQuick PCR extraction kit, repaired at the end and added to PolyA. Polymerase chain reaction amplification (PCR) and sequencing were carried out with an Illumina HiSeq2500, and then the transcriptome data were assembled and annotated. The cDNA libraries of CK and BL (three replicates each) were constructed, sequenced and evaluated. The above processes were conducted by Gene Denovo Biotechnology Co (Guangzhou, China).

2.3. Differentially Expressed Genes (DEGs) and Relationship Analysis of Samples

The genes with FDR < 0.05 and |log2FC| > 1.5 were defined as significantly differentially expressed genes by DESeq2 software (v1.20.0) [19]. GO and KEGG enrichment analyses were used for data screening. NBS class and other disease-resistance-related genes were screened according to the comparative transcript data. Comprehensive analysis was carried out according to the following steps: (1) Pearson correlation coefficient used to evaluate the repeatability of experimental samples. (2) Selection of DEGs (FDR < 0.05 and |log2FC| > 1.5). (3) GO function analysis of DEGs. (4) KEGG enrichment analysis of DEGs. (5) Screening of disease-resistance-related DEGs.

2.4. Fluorescence Quantitative PCR Analysis

Fifteen DEGs were randomly selected, and gene-specific primers were designed for quantitative real-time PCR (qRT-PCR) analysis to detect the gene expression level and verify the accuracy of transcriptome data. The total volume of the reaction system was 20 µL, containing 1 µL diluted cDNA, 400 nM of each primer and 10 µL of the 2 × SYBR real-time PCR premixture (Vazyme), with the following cycling conditions: 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 60 °C for 30 s. After the reaction, the amplification curves and melting curves were analyzed to verify the specificity of the amplification products. Using U2af as the internal reference gene, relative expression levels were analyzed with the $2^{-\Delta\Delta CT}$ method [20]. Three technical replications were included in both the treatment group and the control group. One-way ANOVA with the least significant difference (LSD) test was used for statistical analysis. When $p < 0.05$, the value is considered to be statistically significant. All figures were created using the Origin 2021 software.

3. Results and Analysis

3.1. Infection and Identification of PWN in Black Pine Seedlings

The infection experiment was successful, and on the third day after infection, PWN left the infection site and began to transfer. On the fifth day after infection, PWN were distributed in stem and lateral branches of black pine seedlings. Therefore, needles were collected as samples on the fifth day after infection for subsequent transcriptome sequencing analysis. After inoculation with PWN, the black pine trees gradually wilted with the extension of time. After 45 days of inoculation, the seedlings in CK remained healthy without changes in needles (Figure 1A), while more than 85% of the black pine seedlings in BL had yellow or withered needles (Figure 1B).

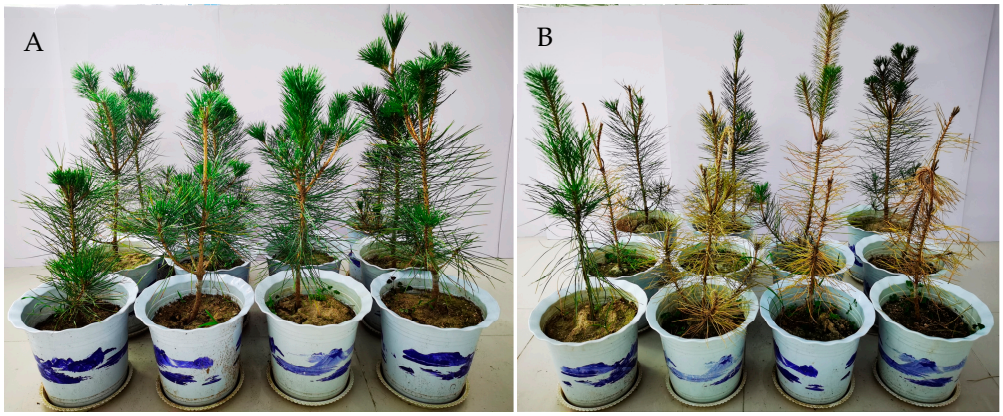

Figure 1. Effects of PWN infection on black pine seedlings. (**A**) The control group (CK). (**B**) The treatment group (BL).

3.2. Transcriptome Sequencing and Assembly

Q20 and Q30 are the key parameters of sequencing base quality.

Q20 represents the percentage of bases with a base recognition rate of ≥99% in Clean-Data, and Q20 ≥ 97% in each replicate. Q30 represents the base percentage of ≥ 99.90%. The more balanced the base composition of each group with Q30 ≥ 93%, the higher the quality, and the more accurate the subsequent analysis. As shown in Table 1, the minimum value of Q20 is 97.84%, and the minimum value of Q30 is 93.80%, indicating that the sample meets the quality requirements of the base.

Table 1. Analysis table of base mass.

Sample	Raw Data (bp)	Clean Data (bp)	AF_Q20 (%)	AF_Q30 (%)	AF_GC (%)
CK-1	7,154,388,900	7,097,563,080	98.04	94.32	45.13
CK-2	7,086,201,600	7,030,845,840	98.12	94.52	45.24
CK-3	7,342,288,200	7,287,440,931	98.00	94.23	45.10
BL-1	7,096,215,600	7,039,809,015	97.84	93.80	45.35
BL-2	6,642,519,000	6,587,501,867	98.08	94.36	45.36
BL-3	7,055,390,400	6,993,540,884	98.02	94.28	45.21

The total number of assembled universal genes (unigenes) was 101,085, with an average length of 820 bp. Most of the length of the unigenes was distributed between 201 bp and 3000 bp, and the frequency of unigenes with a length of less than 1000 bp was the highest. Benchmarking Universal Single-Copy Orthologs (BUSCO) (http://busco.ezlab.org/ (14 May 2020) was used to evaluate the integrity of the transcriptome assembly. The unigenes were compared with the conserved sequences in total BUSCO group genes (1440). It is shown that the number of complete BUSCO (c) genes is 1157, including 1082 complete and single-copy genes and 75 complete and duplicated genes, accounting for 75.1% and 5.2% of the total BUSCO group genes, respectively. The Q30 value and complete BUSCO (c) analysis show that the transcriptome sequencing results are relatively reliable and can be further analyzed.

3.3. Basic and Advanced Annotates of Unigenes

A total of 44,120 (43.65%) genes were successfully matched with known genes in public databases, and approximately 39,747 (39.3%), 35,386 (35%), 27,248 (27%) and 32,152 (31.8%) unigenes were annotated in the Non-Redundant Protein Sequence Database (Nr), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG) and Swiss Protein (Swiss-Prot) databases, respectively. A total of 21,237 unigenes were annotated simultaneously in these four databases (Figure 2).

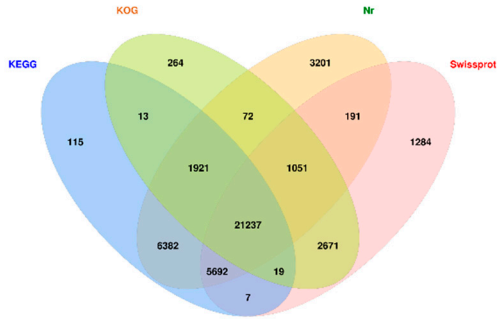

Figure 2. Annotated Venn diagram of unigenes in four databases.

In our study, the predicted protein sequences were compared with the corresponding Transcription factors (TF) database (plant tfdb/animal tfdb) with hmmscan. A total of 1046 transcription factor genes were detected. The statistical results of TF classification families are shown in Figure 3. The top 10 TF families in terms of the number of unigenes are C2H2 (94), MYB_related (93), bHLH (82), ERF (71), NAC (61), MYB (52), C3H (51), WRKY (50), bZIP (45) and Trihelix (43).

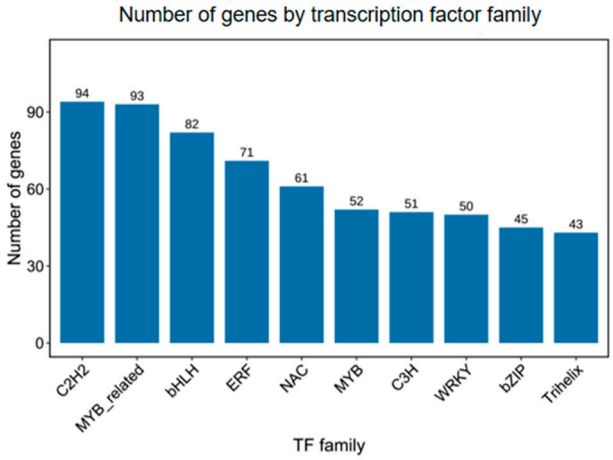

Figure 3. Transcription factor (TF) family statistics. The abscissa is the name of the transcription factor families, and the ordinate is the number of genes.

According to the plant resistance (R) gene database, a total of 3352 candidate R genes were identified in the black pine transcriptome. According to the different conserved domains, the R genes can be divided into different categories, in which the gene numbers of KIN (related domain Kinase, TM), L (LRR), N (NBS, TM), RLP (LRR, TM), T (TIR, TM) and TN (NBS, TIR, TM) are 1229, 664, 557, 316, 227 and 111, respectively, and the others are less than 100, or are even single digits (Figure 4).

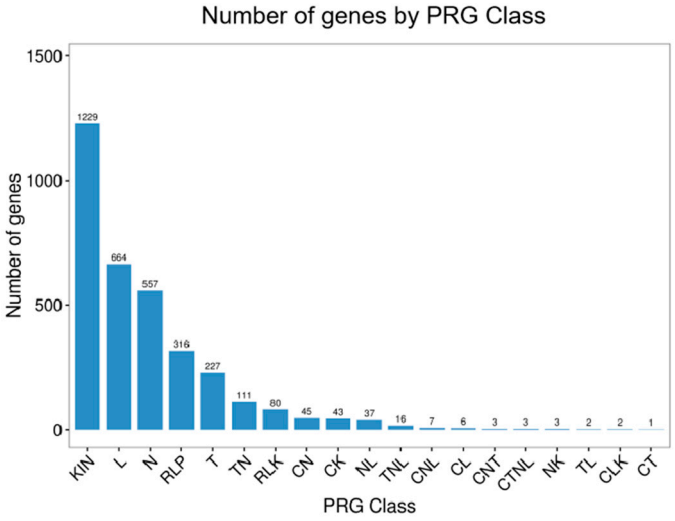

Figure 4. Statistical chart of the R gene classification family. The abscissa is the name of the R gene families, and the ordinate is the number of genes.

3.4. Differential Gene Analysis

We take the expression quantity of any two samples, calculate the Pearson correlation coefficient between these two samples, and then show the correlation between the two samples in the form of a thermal map. It can be seen from Figure 5 that the repeatability between samples within the group is reasonable.

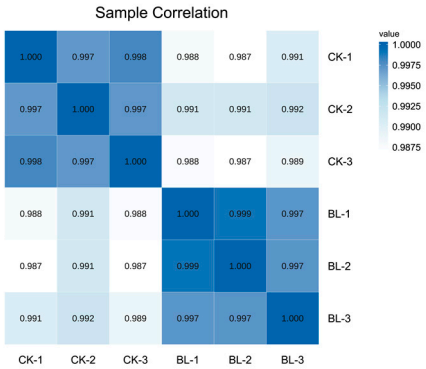

Figure 5. Sample correlation heat map. The abscissa and ordinate are each sample, and the color depth indicates the correlation coefficient between the two samples.

Based on the results of inter-group difference analysis, we screened the genes with FDR < 0.05 and |log2FC| > 1.5 as significantly differentially expressed genes. There were 647 DEGs, of which 277 were significantly upregulated and 370 were downregulated (Figure 6).

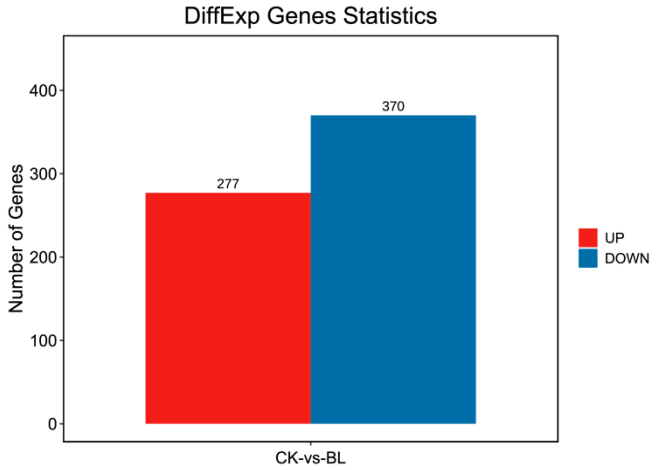

Figure 6. Differential gene statistical map. Red represents upregulated DEGs, blue represents downregulated DEGs.

The results of GO functional annotation and classification statistics show that there are DEGs in three ontologies (biological processes, cell components and molecular function). In biological processes, the DEGs are mainly concentrated in the categories of metabolic process (67 up, 81 down), cellular process (65 up, 64 down), single-organism process (57 up, 51 down) and response to stimulus (24 up, 20 down). However, there are no upregulated genes in the cell killing and locomotion categories. Among the cell components, the DEGs are mainly distributed in cell (25 up, 36 down) and cell part (25 up, 36 down), and in the organelle, macromolecular complex and extracellular region, the number of downregulated genes is significantly higher than that of upregulated genes. In the three categories of virion, virion part and supramolecular fiber, there are no downregulated genes. Among the genes involved in molecular function, the significant DEGs are mainly distributed in binding (80 up, 58 down) and catalytic activity (72 up, 73 down), and there are no upregulated genes in antioxidant activity, electron carrier activity, molecular function regulator, molecular transducer activity and signal transducer activity (Figure 7). PWN infection may cause the differential expression of major functional genes in black pine needles, thus affecting the resistance of black pine seedlings.

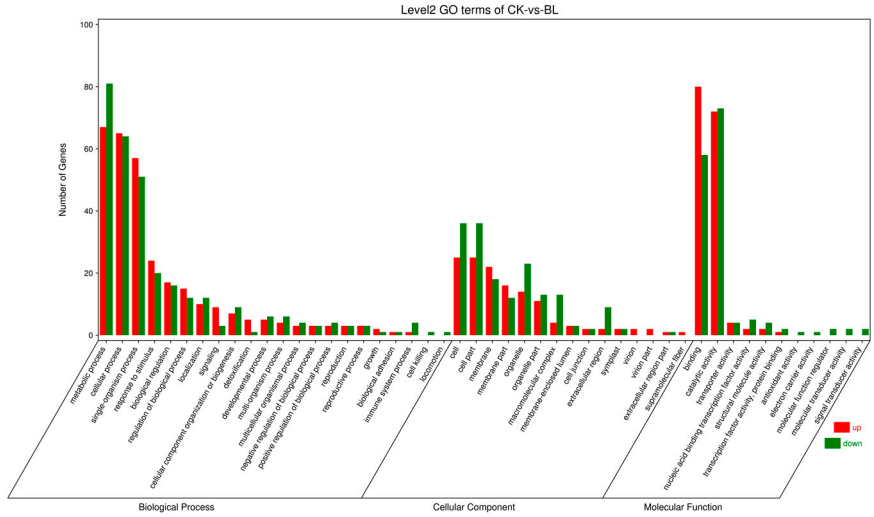

Figure 7. Histogram of GO enrichment and classification of differential genes. The abscissa is the secondary GO terms, and the ordinate is the number of differential genes in the term. Red indicates upregulation and green indicates downregulation.

In our study, 647 DEGs were annotated into 58 metabolic pathways, and Figure 8 shows the 20 most representative pathways. Among them, DEGs show significant differences in the thiamine metabolic (ko00730), biosynthesis of secondary metabolites (ko01110), carotenoid biosynthesis (ko00906), flavonoid biosynthesis (ko00941), terpenoid backbone biosynthesis (ko00900) and plant–pathogen interaction (ko04626) categories, and the number of differential genes accounts for the largest proportion in biosynthesis of secondary metabolites (32) and metabolic paths (41). The significant enrichment analysis based on KEGG pathways showed that PWN infection affected the secondary metabolism of black pine needles; these metabolites may be the main chemical defense substances in plants.

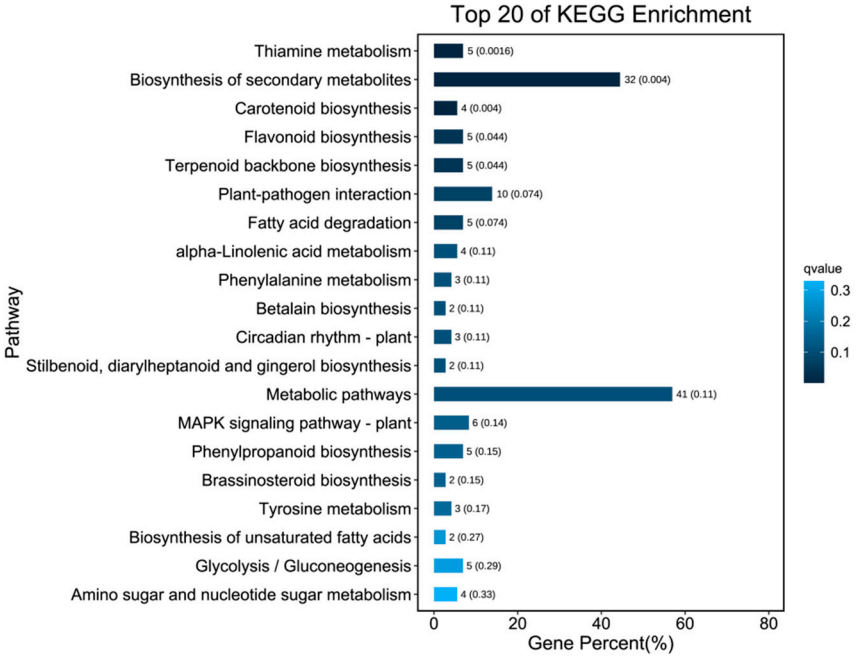

Figure 8. KEGG enrichment bar chart. The first 20 pathways with the lowest *Q* value are used to draw the map, the ordinate is pathway, the abscissa is the proportion of the pathway number in all differential genes; the darker the color, the smaller the *Q* value, and the value on the column is the pathway number and *Q* value.

The annotated high-quality unigenes provide a basis for the further analysis of the DEGs and the metabolic pathways of black pine infected with PWN.

3.5. PCR Analysis Results

Twelve DEGs were randomly selected, and gene-specific primers were designed for qRT-PCR analysis to detect the gene expression level and verify the accuracy of transcriptome data (Table 2). It is shown that the DEGs proved by qRT-PCR were highly consistent with the results of transcriptome sequencing analysis (Figure 9). Specifically, ten unigenes, including unigene0001984, unigene0083685, unigene0033503, unigene0000278, unigene0048705, unigene0083090, unigene0003367, unigene0035857, unigene0005880 and unigene0020248 were downregulated, and two unigenes, including unigene0074724 and unigene0012357, were upregulated. Based on the above analysis, the qRT-PCR analysis results are considered to support the reliability of the sequencing data. The difference between groups may be related to the batch difference between samples.

Table 2. Primers used for qRT-PCR.

ID	Primer Name	Sequence (5′to 3′)
Unigene0001984	Unigene0001984-F	CAATCAATGGAGCAGTAGA
	Unigene0001984-R	GCAATAGGTGGTGTAATGA
Unigene0083685	Unigene0083685-F	CATCTTCTGCTGTTCAATC
	Unigene0083685-R	GCTCTTCATCAAAGTTACC
Unigene0033503	Unigene0033503-F	GGATTCTACACTTACAATGC
	Unigene0033503-R	CATAACATTAGCGAAGAAGG
Unigene0000278	Unigene0000278-F	CGCTGGTTGAATATACTCT
	Unigene0000278-R	GGATATAGGTAGGCATCTG
Unigene0048705	Unigene0048705-F	CTTGTAACAGCAGAGGAG
	Unigene0048705-R	CAATACCAGAGAGGGAAAT
Unigene0083090	Unigene0083090-F	CCTCTTGAATTGCCTCAT
	Unigene0083090-R	GACCAACAACCATTATGC
Unigene0003367	Unigene0003367-F	CTGAAAGTGACTGATGGA
	Unigene0003367-R	TTGGAATAGTTCGTAGGAG
Unigene0035857	Unigene0035857-F	GCTTCTGGACTCTTTCAA
	Unigene0035857-R	CTGTCTGTTCTCCTCCTA
Unigene0005880	Unigene0005880-F	ACCTGAGAAGAAGAAGATTC
	Unigene0005880-R	GCAAAGAGCCTATGGATT
Unigene0020248	Unigene0020248-F	TGCTCTGGCTTCCATCGTTT
	Unigene0020248-R	CTGGTTTCCCGAGCATCACT
Unigene0074724	Unigene0074724-F	ATGCGAGGGATTGCTCCTTG
	Unigene0074724-R	CTGTGGAGCCCCATGAACTG
Unigene0012357	Unigene0012357-F	CGTATGAGCCCCAGTATGCC
	Unigene0012357-R	GGTCCCATCTTCTCACAGCC
U2af	U2af-F	TCGGGAGGTTGGGTCTACAT
	U2af-R	ACCAGTCCTTCAGTCCCCTT

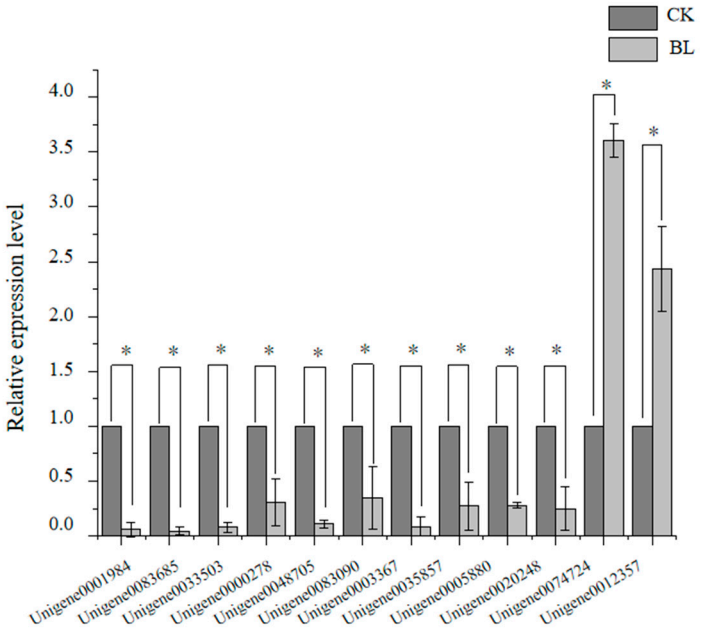

Figure 9. DEGs qRT-PCR analysis. The abscissa represents DEGs, and the ordinate represents the relative gene expression level obtained by real-time PCR (*: $p < 0.05$, the difference was statistically significant).

3.6. Screening Disease-Resistance-Related DEGs

When resisting pathogens and adverse environmental conditions, R genes are activated in plants [21]. There are three R genes (unigene0059253, unigene0007068 and unigene0035790) and six with significant differences in expression (unigene0043826, unigene0069489, unigene0031688, unigene0003092, unigene0085494 and unigene0002084), belonging to the NBS (nucleotide-binding site) class and LRR (Leucine-rich repeat) receptor-like serine/threonine-protein kinases class, respectively (Table 3). It is speculated that R genes participate in the immediate regulation of disease resistance when black pine is infected by PWN.

Table 3. Screening of differential R genes.

ID	log2(fc)	Description
Unigene0059253	1.6229304	TIR/NBS/LRR disease resistance protein
Unigene0007068	5.6885001	TIR-NBS-LRR protein
Unigene0035790	1.6295281	TIR-NBS-LRR protein
Unigene0043826	1.9064777	LRR receptor-like serine/threonine-protein kinase EFR
Unigene0069489	4.5993619	LRR receptor-like serine/threonine-protein kinase At3g47570
Unigene0031688	7.5062084	LRR receptor-like serine/threonine-protein kinase At1g56140 isoform X1
Unigene0003092	10.81645	LRR receptor-like serine/threonine-protein kinase At3g47570
Unigene0085494	10.70275	LRR receptor-like serine/threonine-protein kinase At3g47570
Unigene0002084	−2.057195	LRR receptor-like serine/threonine-protein kinase At3g47570 isoform X6

In the defense response of plants to pathogen infection, transcription factors play an important role by regulating the orderly transcription of signal pathways. There are four common types of plant transcription factors related to disease resistance: ERF [22], MYB [23], WRKY [24] and bZIP [25]. Among the top 10 TF families in terms of the number of unigenes, 71, 52, 50 and 45 disease-resistant genes were identified in the four transcription factor families, respectively. A total of three, three and one significant DEGs were identified in ERF, MYB and WRKY, respectively (Table 4). Therefore, it is speculated that the transcription factors ERF, MYB, and WRKY participate in the plant defense response to PWD through signal transduction and other pathways.

Table 4. Screening of differential transcriptional factor genes.

TF Families	Description
ERF	unigene0084830, unigene0028109, unigene0072376
MYB	unigene0100697, unigene0040542, unigene0073218
WRKY	unigene0046254

The thiamine metabolism pathway participates in various cellular responses in plants and plays an indispensable role in disease resistance, stress resistance and crop yield [26]. It consists of three enzymes: phosphomethylpyrimidine synthase (THIC), thiamine phosphate phosphatase (TH2) and thiamine pyrophosphokinase (TPK2). Thiamine is also related to the activity of Phenylalanine ammonialyase (PAL), which further affects the synthesis of resistant substances such as total phenols, flavonoids and phytochemicals [27,28]. Here, one, two, two and two significant DEGs were identified as encoding TPK2, THIC, TH2 and PAL, respectively (Table 5). This means that they are important to improving the disease resistance of black pine.

Table 5. Screening of differential enzyme genes.

ID	log2(fc)	Description
Unigene0010372	11.859793	Putative thiamine pyrophosphokinase
Unigene0020296	12.303019	Phosphomethylpyrimidine synthase
Unigene0027776	10.550746	Phosphomethylpyrimidine synthase
Unigene0060145	12.404964	Thiamine phosphate phosphatase/amino-HMP aminohydrolase
Unigene0089674	6.915879	Thiamine phosphate phosphatase/amino-HMP aminohydrolase
Unigene0020248	−1.546972	Phenylalanine ammonia-lyase
Unigene0079836	10.090112	Phenylalanine ammonia-lyase
Unigene0010629	−1.785339	Aldehyde dehydrogenase family 2 member B7

Aldehydes are highly active molecules which are toxic to cells [29]. The balance of aldehydes in plants is mainly dependent on NAD(P)+ acetaldehyde dehydrogenase (ALDH), which oxidizes aldehydes to carboxylic acids [30]. According to the transcriptome data, unigene0010629 encodes the ALDH2B4 enzyme, and it has downregulated expression (Table 5). The balance between acetaldehyde production and metabolism is disrupted. Excessive acetaldehyde may lead to the metabolic disorder, lipid peroxidation and cell death of black pine, reduction in the defense response of black pine, and intensification of the severity of PWN infection. In a word, the downregulation of *ALDH* gene is probably a major obstacle for black pine in resisting PWD.

4. Discussions

PWD is a major threat to natural forest and ecological environment construction in China [31,32]. Early studies were mainly focused on aspects of PWN such as pathogenicity, PWN-associated bacteria and the microbiome associated with susceptible pine [33–35]. In recent years, research has focused on the response of pine trees to PWN infection. Genome sequencing results of some related species have been reported, including *P. densiflora* [36], *P. pinaster* [37] and *P. massoniana* [38]. In our study, black pines treated with PWN were used as the treatment group and those treated with sterile water as the control. Transcriptome sequencing analysis was carried out, and DEGs and their metabolic pathways related to the PWN resistance of black pine were screened, analyzed and further discussed.

R genes play an important role in plant disease detection and the defense response. Among them, *NBS-LRR* genes, and the corresponding receptor proteins NBS and LRR, are the most dominant [39]. According to the different amino terminal domains, NBS-LRR proteins can be further divided into two categories: TIR-NBS-LRR, which contains the Toll/Interleukin-1 receptor domain, and CC-NBS-LRR, which contains the curl (CC) helical domain [40,41]. In the transcriptome sequencing results of black pine, there were

many disease-resistant genes containing the NBS domain, among which three upregulated *TIR-NBS-LRR* genes were significantly differentially expressed: unigene0059253, unigene0007068 and unigene0035790.

To date, abundant *NBS-LRR* genes have been detected in *Arabidopsis*, *alfalfa*, *poplar*, rice, cassava and sweet potato [42–47]. Some studies have shown that the expression of the *MdNBS-LRR1* gene in apple was upregulated after infection by the ring rot pathogen, and the expression level was 10.6 times higher than that of the control 24 days after inoculation [48]. Zheng et al. determined that the infection of powdery mildew can promote the upregulation of *NBS-LRR* genes in camphor trees [49]. Liu et al. indicated that the expression of *NBS-LRR* genes in the defense response of dragon spruce needle drop disease increased significantly [50]. In our study, the expression of unigene0059253, unigene0007068 and unigene0035790 in the treatment group was significantly higher than that in the control group. Therefore, we speculate that *NBS-LRR* genes may be involved in regulating the defense response of black pine against PWN infection.

In the thiamine metabolism pathway, firstly, THIC catalyzes the biosynthesis of hydroxymethylpyrimidine pyrophosphate (HMP-PP) and condenses hydroxythiazole phosphate (HET-P) in chloroplasts to form thiamine monophosphate (TMP) [51,52]. Then, TH2 encodes a specific TMP phosphatase, which dephosphorylates TMP to thiamine [53]. Finally, thiamine is converted into thiamine diphosphate (TDP) by TPK2 in the cytoplasm [54]. In our study, five genes related to the three enzymes of the thiamine metabolism pathway were found (unigene0020296, unigene0027776, unigene0060145, unigene0089674, and unigene0010372), and the gene expression was significantly upregulated.

Previous studies have shown that TDP is a necessary coenzyme for many central metabolic enzymes [55], such as transketolase (TK) in the Calvin cycle and α-ketoglutarate dehydrogenase (α-KGDH) in the trichloroacetic acid cycle, which are involved in photosynthesis and respiration, respectively. TDP can also directly change the rhythms of plant metabolites [56]. THIC is the only known nucleic acid switch in plants, which regulates the balance of TDP abundance [57]. It can be speculated that the expression of genes encoding TPK2 and THIC in black pine infected with PWN is upregulated, which may play a role in the carbon and nitrogen assimilation balance and health of black pine. Thiamine, also known as vitamin B1, can improve the resistance of wheat, peas and millet to pathogenic fungi infection [58–60]. The probability of rice becoming infected with bacterial blight will increase if the content of thiamine decreases [61]. Thiamine can also increase the activity of PAL and peroxidase (POD) and the content of total phenols, flavonoids and lignin by activating the host defense reaction [62], affecting the disease development of melon fruit [27]. In our study, the expression of *THIC*-, *TH2*- and *TPK2*-related genes increased at the same time, indicating that they are likely to participate in the resistance process of black pine to PWD.

PAL is the key enzyme of phenylpropane metabolism, catalyzing the deamination of L-phenylalanine to produce trans-cinnamic acid and coumarin coenzyme A, and playing a crucial role in the synthesis of flavonoids, lignin and other substances [63]. For example, flavonoids originating from the shikimic acid pathway (phenylpropanoid metabolic branch) [64] have important functions such as antioxidation and free radical scavenging [65]. Phenolic compounds are produced through the cinnamic acid pathway, and the oxidation of phenols is related to plant browning and hybrid death [66]. The formation and accumulation of lignin are closely related to physiological activities such as plant cell differentiation and disease and pest defense [67]. The *PAL* gene also plays an important role in many crops. Yang et al. discovered the key role of the *PAL* gene in cotton resistance to verticillium wilt [68]. Joe et al. proved that PAL was related to the stress and drought resistance of *Lycium barbarum* [69]. Sun et al. studied the disease resistance and stress resistance functions of *PAL* genes, providing a reference for molecular breeding in *Cucurbitaceae* crops [70]. In the black pine samples treated with PWN, the *PAL* gene unigene0079836 was significantly up-regulated, with a difference of a multiple greater than 10, whereas the expression of unigene0020248 was downregulated. The increased expression of *PAL* genes contributes to

the biosynthesis of flavonoids, lignin, phytoalexin and other substances, and may improve the inhibition of PWN, thus improving the resistance of black pine to PWD. However, the role of phenylpropanoid metabolism in plant disease resistance is mainly attributed to the toxicity of disease-resistant substances to pathogens [71]. The continuous accumulation of some direct or indirect metabolites may cause metabolic disorder or toxicity and accelerate the death of black pine.

In summary, *NBS-LRR* genes, thiamine metabolic enzyme genes, *PAL* genes and *ALDH* genes are key genes for the resistance and detoxification of black pine, with important roles in the resistance of black pine to PWN infection. Therefore, studying these genes and their metabolic mechanisms is of guiding significance for the further study of PWD resistance in black pine.

5. Conclusions

In our study, the needles of black pine seedlings were taken as samples for transcriptome sequencing analysis on the fifth day after being infected with PWN. Compared with the control group, 647 genes were differentially expressed in the treatment group, of which 277 genes were upregulated and 370 genes were downregulated. Genes related to PWN resistance were screened, and metabolic pathway analysis was carried out. This study will provide a reference for the study of molecular mechanisms of resistance and the breeding of disease-resistant black pine.

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