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Integrated transcriptomic and metabolic analyses reveal the early response mechanism of *Pinus tabulaeformis* to pine wood nematodes

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Abstract

Pine wilt disease (PWD) is a devastating disease of pine trees caused by the pine wood nematode (*Bursapherenchus xylophilus*, PWN). To study how *Pinus tabulaeformis* responds to PWD infection, we collected 3-year-old *P. tabulaeformis* seedlings at 2 days, 5 days, and 8 days after being infected with *B. xylophilus*. We identified genes and metabolites early responding to infection using transcriptome and metabolomic data obtained by high-throughput mRNA sequencing (RNA-seq) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assays, respectively. The following results were obtained: (1) After inoculation with PWN, the average number of days taken for 3-year-old *P. tabulaeformis* seedlings to develop symptoms was 8 days. (2) Combined transcriptome and metabolome analysis revealed that phenylpropanoid biosynthesis and flavonoid biosynthesis are critically important pathways for *P. tabulaeformis* to respond to PWD. (3) The response of *P. tabulaeformis* to stress was mainly through positive regulation of gene expression, including some key genes related to plant hormones or transcription factors that have been widely studied. Genes related to pathways such as photosynthesis, plant-pathogen interactions, and DNA replication were downregulated. (4) Terpenoid biosynthesis genes involved during the development of pine wilt disease. This study demonstrated the defence and pathogenic mechanisms of *P. tabulaeformis* against PWD, providing a reference for the early diagnosis of PWD.

Keywords Pine wilt disease, *Bursaphelenchus xylophilus*, Phenylpropanoid, Flavonoid, Transcriptomic, Metabolic

Introduction

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) originates in North America and is the leading cause of pine wilt disease (PWD) [1]. PWD is a complex disease system composed of a variety of organisms that cause serious harm in Asia, especially in China and Japan, resulting in widespread death of pine trees. Currently, 52 countries have regarded it as a plant quarantine disease. The pathogenic mechanism of PWN is complex. There are 3 common views on the pathogenic mechanism: First, previous studies have shown that the blockage of water conduction caused by tracheid cavitation is a critical factor resulting in the death of infected pine trees. Second, cellulase excreted by PWNs is the pathogenic substance of PWD and is responsible for the development of early symptoms. Third, many toxic substances have accumulated in nematode-infected pine trees. For preventing

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the spread of PWD, the main methods currently used are strict quarantine and control of insect vectors that transmit the disease. Analyzing the early response mode of pine trees after PWN infection can help us study the molecular mechanism of pathogenesis, screen resistance genes, and cultivate transgenic resistant plants by gaining information on differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs). In addition, the study of indicators of early infection can help us detect the disease as soon as possible and carry out treatment on time.

With the development of transcriptome sequencing technology, an increasing number of researchers have begun to analyse the gene response mode of pine materials such as *Pinus massoniana* and *Pinus densiflora* to PWD. Previous studies have shown that the molecular defence responses produced by pine trees after PWN infection include activation of the oxidative stress response, cell wall lignification, and biosynthesis of terpenoids and phenylpropanoids [2]. Studies have shown that in infected *P. massoniana*, the pathways of genes involved in phenylpropanoid metabolism and flavonoid biosynthesis are enriched, the levels of total phenols and total flavonoids are reduced, the auxin response family proteins are downregulated, the genes related to pathogen recognition, PWD resistance, and growth regulation are downregulated, and the levels of phytoalexin-like secondary substances are reduced, leading to withering and eventual death of *P. massoniana* [3]. And flavonoid biosynthesis is more highly expressed in resistant pine than in susceptible pine, and phenolic metabolites were produced more in resistant pine [4]. The expression of genes related to antimicrobial peptides and putative pathogenesis-related genes is much higher in susceptible trees than in resistant trees, while cell wall-related genes are higher in resistant trees than in susceptible trees [5]. Compared to uninoculated trees, PWN-inoculated trees had higher expression levels of genes encoding pathogenesis-related proteins, pinosylvin synthases, and metallothioneins [6].

In addition to transcriptomics research, metabolomics has been applied to research. A nontargeted metabonomic study based on liquid chromatography-mass spectrometry (LC-MS) technology on pine trees inoculated with PWN was conducted, and the DAMs were mainly enriched in pathways such as biosynthesis of secondary metabolites and flavonoid biosynthesis [7]. Different from susceptible *P. pinaster*, small changes were observed in the main metabolites of resistant plants, such as alanine, citrate, fructose, galactinol, glucose, glutamate, malate, myo-inositol, phenylalanine, and quinate [8]. And in the susceptible phenotype, significant increases in salicylic acid (SA) and methyl jasmonate (JA-ME) were observed, indicating that the higher susceptibility of pine trees to PWN infection may be due to the inefficient triggering of the hypersensitive response in which the JA and SA pathways are involved [9].

In this study, 3-year-old *P. tabulaeformis* was used as the research material, and samples were taken at different time points before the onset of symptoms after infection. From the perspectives of transcriptomics and metabolomics, the early response of *P. tabulaeformis* to PWNs was explored and the defence mechanism and pathogenic mechanism of pine against PWNs were studied, providing a reference for the early diagnosis of PWD.

Materials and methods

Observation of the onset time of *Pinus tabulaeformis*

A total of 5 strains of 3-year-old *P. tabulaeformis* were used. The PWN (*Bursapherenchus xylophilus*) was taken from *P. tabulaeformis* in Dongling Park, Shenyang City, Liaoning Province. The nematode was isolated and reared with *Botrytis cinerea*. The pipette tip containing the PWN suspension was attached to the top of the trimmed pine trunk, and the connection between the pipette tip and plant was sealed with a film. After inoculation, pine seedlings were cultured under sufficient light at 28 ℃. The number of days it took to observe disease symptoms was calculated.

Transcriptome sequencing and analysis

According to the above experiments, the time required for 3-year-old pine to develop symptoms is 8 days. To investigate the early response mechanism of PWN infection, 12 pine seedlings were harvested on the 2 days, 5 days, and 8 days after inoculation with PWNs, numbered Pt2d, Pt5d, and Pt8d, respectively. The control group was sampled 2 days after inoculation with sterile water and labeled CK, each sample had 3 replicates. The young branches of *P. tabulaeformis* were cut off, immediately placed into liquid nitrogen, stored at -80 ℃, and sent to Genepioneer Biotechnologies for transcriptome and metabolome sequencing.

RNA was extracted and tested for detection of RNA purity and integrity using a NanoDrop 2000 and Agilent Bioanalyzer 2100, respectively. Magnetic beads with oligo (dT) enrich mRNA. After PCR enrichment, a cDNA library was obtained, qualified, and sequenced using the Nova seq6000 platform. The raw reads were filtered to obtain clean data. FastQC was used to evaluate the quality of sequencing data and sequence assembly on clean data to generate unigenes by Trinity [10]. BLAST was used to compare unigene sequences with databases such as Gene Ontology (GO) (http://geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/kegg/pathway.html) to obtain annotation information [11, 12]. $|\text{Log2 (FC)}| \geq 1$ and FDR < 0.05 were used as the criteria for DEG screening using DESeq2 [13]. Mfuzz was used to cluster DEGs.

Heatmap and KEGG enrichment analysis was conducted by TBtools [14].

Analysis of untargeted metabolomics-derived data

The above 12 experimental samples were subjected to LC-MS/MS-based nontargeted metabolomics analysis, including data quality control, data filtering, missing value processing, and normalization processing [15] (Fig S4). The P value of Student's t-test is less than 0.05, and the variable importance in the projection (VIP) of the first principal component of the orthogonal partial least squares-discriminant analysis (OPLS-DA) model is greater than 1 [16]. The KEGG PATHWAY database was used to conduct pathway analysis on different substances [17].

qRT-PCR validation

To verify the reliability of the transcriptome sequencing data, 12 genes were selected randomly from DEGs, and reverse transcription was performed on the extracted RNA using a HiScript 1st Strand cDNA Synthesis Kit. AceQ qPCR SYBR Green Master Mix (without ROX) was used for fluorescence quantitative PCR, and three replicates were set. The relative expression level of genes was calculated using the $2^{-\Delta\Delta CT}$ method.

Results

The onset time of pine Wilt disease in 3-Year-old pinus tabulaeformis has been determined

To clarify the disease progression of *P. tabulaeformis* seedlings in response to PWN infection, 5 seedlings were inoculated with PWNs, and their phenotypes were observed. Disease symptoms first appeared at the base of the needle close to the inoculation site, showing a phenomenon of fading green and yellowing (Fig. 1-A). The specific onset time of disease for 5 seedlings is shown in Table 1, diseased needles were observed for 8.2 days on average after inoculation. After one month of inoculation, the number of coniferous needles that showed disease symptoms gradually increased, and the newly grown unwooded twigs drooped. The colour of the *P. tabulaeformis* needles turned white and gradually became dehydrated and withered (Fig. 1-B). With increasing time after inoculation, the yellow needles gradually increased. Two months after inoculation, the whole *P. tabulaeformis* seedling dried up and the needles showed signs of gradually turning red (Fig. 1-C). Finally, the *P. tabulaeformis* seedlings died. In the later stage of *Pinus* infection with PWNs, symptoms similar to those of drought stress in *Pinus* were observed, which may be due to the large-scale reproduction of PWNs that prevented plants from absorbing water normally.

Differentially expressed gene analysis

According to the information average onset time of the disease after inoculation with PWNs is 8 days, to identify early response genes for PWN infection, we took samples of *Pinus* inoculated with PWNs for 0 day (control group), 2 days, 5 days, 8 days. For each sample, we set up 3 biological replicates for transcriptome sequencing analysis. The quality of sequencing data was shown in the supplementary materials, and the genome was assembled properly for subsequent analysis (Table S3, S4 and Fig S1, S2). 13 early response DEGs were selected for qRT-PCR verification. As shown in Fig S3, the trends of qRT-PCR and transcriptome data were consistent, providing that the transcriptome sequencing results were reliable.

The number of DEGs in inoculated pine trees compared with untreated CK is shown in Fig. 2-A. With increasing inoculation time, the number of DEGs gradually increased. Compared with CK, there were 1013 DEGs (639 up- and 374 downregulated) after inoculation for 2 days and 2285 DEGs (1314 up- and 971 downregulated) after inoculation for 5 days, while after inoculation for 8 days, the number of DEGs significantly increased to 8728 (5071 up and 3657 down), which shows that the inoculation time has a huge impact on *Pinus*. From the results, it can also be seen that *Pinus* responded to PWN infection mainly through positive regulation during the inoculation process (The number of upregulated genes was greater than that of downregulated genes in CK vs. Pt2d, CK vs. Pt5d, CK vs. Pt8d).

A Venn diagram was used to compare the distribution of DEG numbers among different groups, as shown in Fig. 2-B. Compared with CK, 541, 773, and 7126 DEGs were specifically expressed after inoculation for 2 d, 5 d, and 8 d, respectively, indicating that *Pinus* has different response mechanisms at the mRNA levels as the inoculation time increased. There were 272 DEGs differentially expressed at all inoculation time points. We perform KEGG enrichment on the parts of 272 genes with | Log2 (FC) | ≥ 2, found that significant enrichment in amino sugar and nucleotide sugar metabolism, plant hormone signal transduction, phenylpropanoid biosynthesis, transcription factors, biosynthesis of other secondary metabolites (corrected p value < 0.05, Table S1). The heatmap of DEGs in these pathways are shown in the Fig. 2-C: they are upregulated at 2 d, 5 d, and 8 d compared with CK, including 4 *Jasmonate ZIM domain-containing proteins (JAZ)*, 3 *MYB transcription factor*, 4 *AP2 domain proteins.* The changes in the expression levels of these genes may affect downstream gene expression in hormone pathways or regulate downstream gene expression as transcription factors, which are relatively critical genes.

Fig. 1 Symptoms of *Pinus tabulaeformis* seedlings after inoculation with pine wood nematodes. (**A**) Initial symptoms of *Pinus*, temporal disease symptoms have been defined in red box. (**B**) Symptoms of *Pinus* 1 month after inoculation. (**C**) Symptoms of *Pinus* 2 months after inoculation

Table 1 The onset time of infection in *P. Tabulaeformis*

ID of Pinus tabulaeformis	Days before onset
1	7
2	7
3	8
4	9
5	10
mean value	8.2

KEGG pathway enrichment analysis of differentially expressed genes

To study the expression patterns of early response genes at different time points before the onset of the disease, we identified 10,097 DEGs in at least one comparison and grouped them into 8 clusters using a fuzzy c-means clustering (Fig. 3-A). KEGG enrichment analysis was performed on each cluster of genes (Fig. 3-B). DEGs with various expression patterns in clusters 2, 3, 4, 5, and 7 were significantly enriched in phenylpropanoid biosynthesis (corrected p value < 0.05). DEGs that immediately began to downregulate after 2 days of treatment were significantly enriched in photosynthesis-related pathways including Photosynthesis, Photosynthesis proteins, Photosynthesis -antenna proteins, Carbon fixation in photosynthetic organisms, and Porphyrin and chlorophyll metabolism (Clusters 2, 7). Additionally, DEGs in cluster 7 were significantly enriched in Cutin, suberine and wax biosynthesis, Pentose and glucuronate interconversions, Cytochrome P450, Carbohydrate metabolism, Phagosome, Cyanoamino acid metabolism, Starch and sucrose metabolism, and many chromosomal-related genes. DEGs significantly downregulated during treatment for 2–5 days were enriched in Plant-pathogen interactions (Clusters 3, 6). The continuously upregulated genes were significantly enriched in Fatty acid degradation, Tyrosine metabolism, Pyruvate metabolism, Glycolysis/

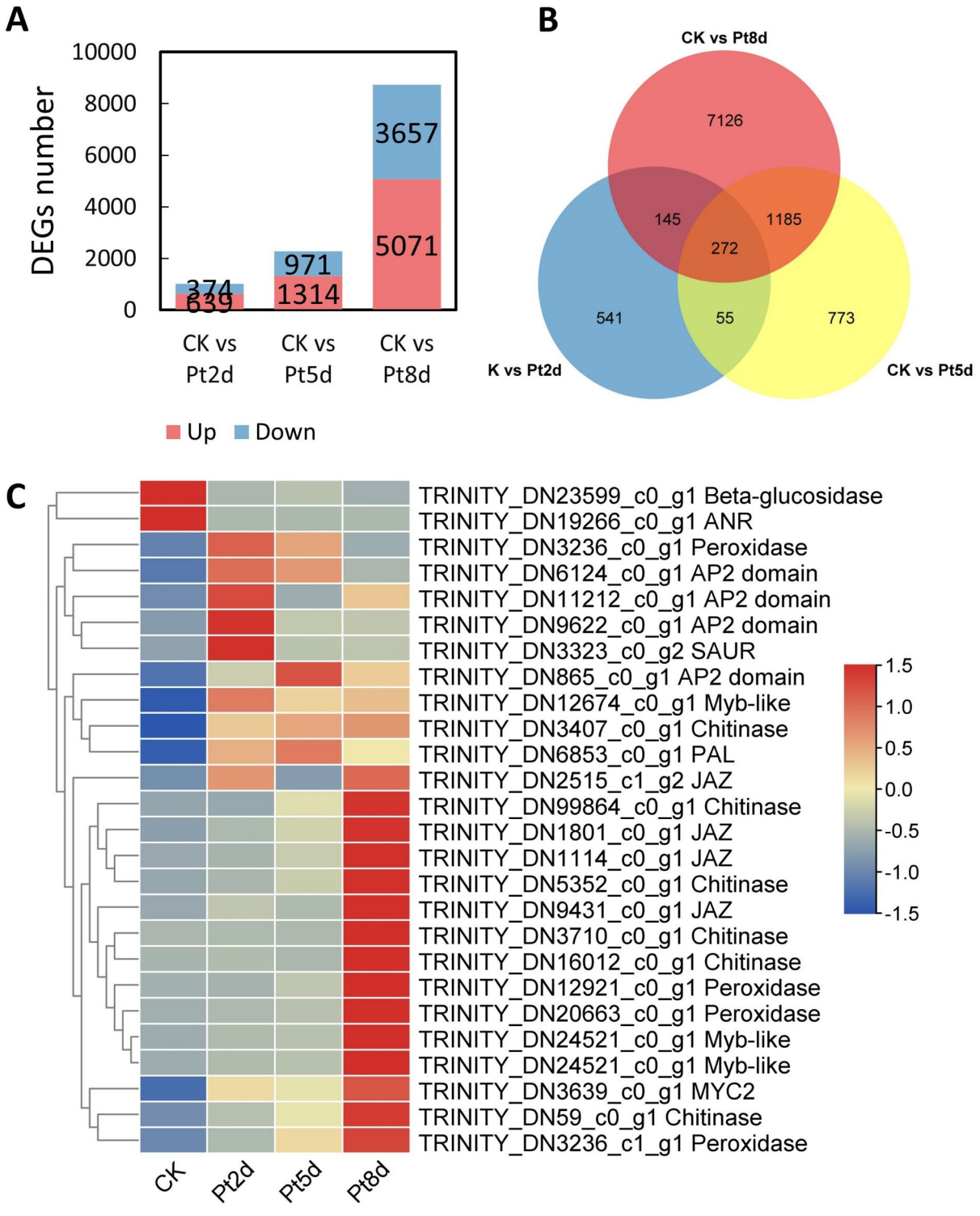

Fig. 2 DEGs in different inoculation times. (**A**) Number of DEGs in different comparisons. (**B**) Venn diagram of DEGs. (**C**) Heatmap of DEGs (|Log2 (FC) | ≥ 2)shared at different treatment times in enriched KEGG pathway

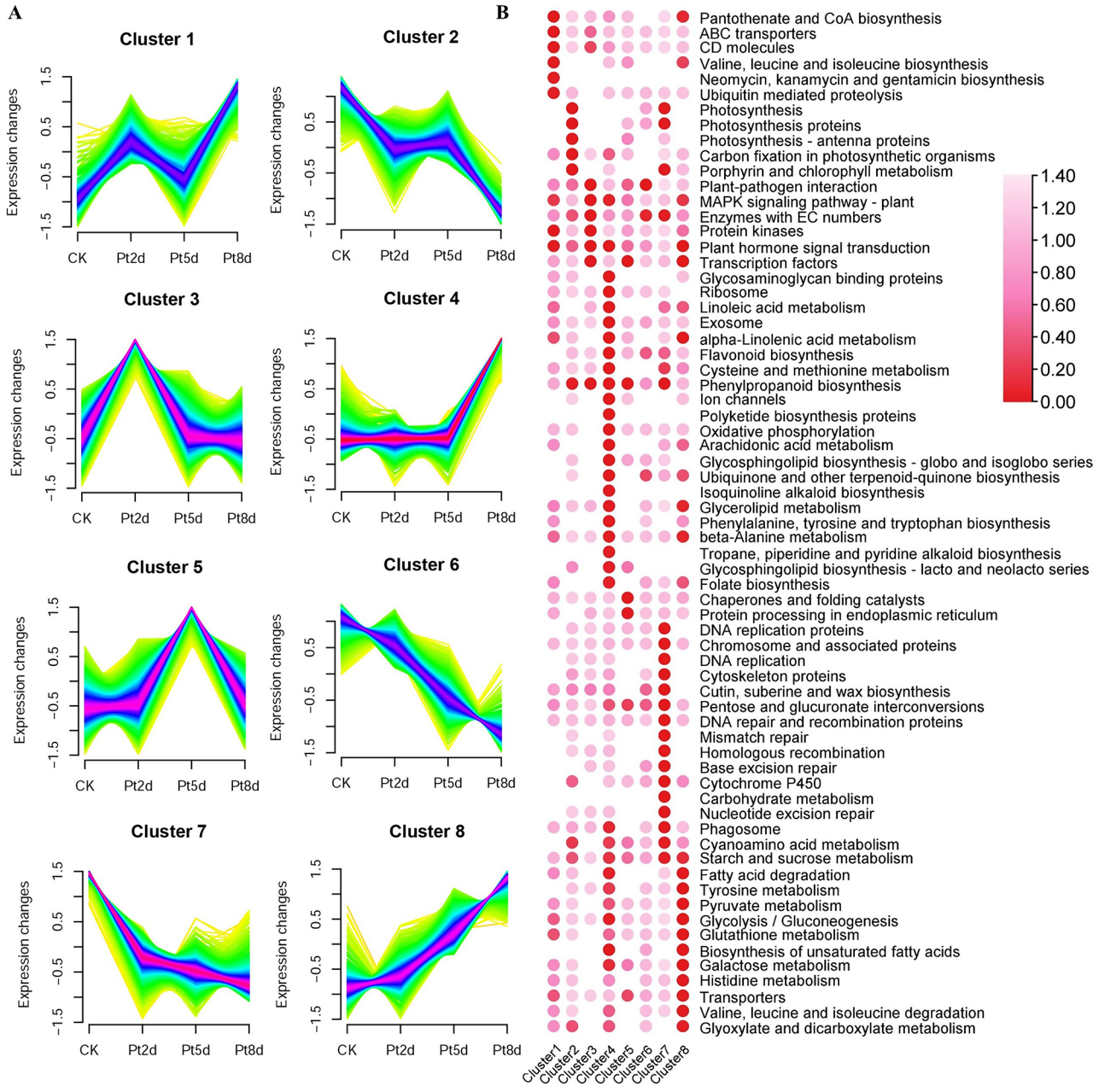

Fig. 3 KEGG enrichment analysis of DEGs. (**A**) Cluster analysis of gene expression patterns at different time points. (**B**) KEGG enrichment analysis of genes with different expression patterns (colour indication corrected p-value, white for a blank value)

Gluconeogenesis, Glutathione metabolism, Biosynthesis of unsaturated fatty acids, Galactose metabolism, Histidine metabolism, Transporters, Valine, leucine and isoleucine degradation, Glyoxylate and dicarboxylate metabolism (Cluster 8). Fluctuate upregulated DEGs were significantly enriched in Pantothenate and CoA biosynthesis; ABC transporters; CD molecules; Valine, leucine and isoleucine biosynthesis; Neomycin, kanamycin and gentamicin biosynthesis; and Ubiquitin-mediated proteolysis (Cluster 1). DEGs that started to be upregulated until 5th day were significantly enriched in Linoleic acid metabolism, Flavonoid biosynthesis, Glycosphingolipid biosynthesis, Polyketide biosynthesis proteins, Oxidative phosphorylation, Arachidonic acid metabolism, Ubiquinone and other terpenoid-quinone biosynthesis, Isoquinoline alkaloid biosynthesis, Glycerolipid metabolism, Tropane, piperidine and pyridine alkaloid biosynthesis, Folate biosynthesis and several amino acid metabolism pathways (Cluster 4). In summary, DEGs at different time stages participated in different biological processes, which showed both consistency and specificity. During this process, related genes such as those

involved in signal transduction, organic metabolism, photosynthesis, plant-pathogen interactions, flavonoid biosynthesis, and phenylpropanoid biosynthesis, were widely expressed, indicating that they play an important role in the early response of *P. tabulaeformis* to PWD.

Analysis of differentially accumulated metabolites after inoculation

Metabolomics analysis based on LC-MS/MS was performed on the same samples. Differentially accumulated metabolites (DAMs) with VIP≥1 and $P<0.05$ were screened. As shown in Figs. 4-A and 39 DAMs (15 up, 24 down) were obtained in the CK vs. Pt2d, 37 DAMs (11 up, 26 down) were obtained in the CK vs. Pt5d, and 52 DAMs (9 up, 43 down) were obtained in the CK vs. Pt8d. The common DAMs in the 3 comparison groups were L-methionine (downregulated), crategolic acid (downregulated), and adenosine 3'-monophosphate (upregulated) (Fig. 4-B, C).

According to VIP≥1 and $P<0.01$, 34 extremely significant differentially accumulated metabolites (DAMs) were screened in the positive and negative ion modes (Fig. 5-A). The super classes that contained DAMs from high to low were: Unclassified, organoheterocyclic compounds, Lipids and lipid-like molecules, Benzenoids, Organic acids and derivatives, Nucleosides, nucleotides, and analogues (Fig. 5-B). According to the KEGG annotations, extremely significant DAMs participated in amino acid synthesis and metabolism pathways, plant secondary metabolism, signal transduction, and vitamin-related pathways (Table S2). Among them, 3-(2,3-dihydroxyphenyl) propanoate is involved in phenylalanine metabolism, and hesperetin is involved in flavonoid biosynthesis. Many studies have indicated that phenylalanine and flavonoids are closely related to plant stress resistance. Dehydroepiandrosterone, indole-3-acetic acid, and L-methionine are involved in the synthesis of plant hormones, which may regulate multiple genes downstream of the pathway. 16-Hydroxy hexadecanoic acid is involved in Cutin, suberine and wax biosynthesis, which can protect plants from invasion by foreign organisms [18]. (2 S,5 S)-trans-carboxymethylproline participates in Carbapenem biosynthesis, which is a class of antibiotics [19]. This shows that the metabolites of *P. tabulaeformis* respond to pathogens at multiple levels.

Integrative analysis of transcriptomics and metabonomics

Integrating analysis of the KEGG enrichment of DEGs and DAMs revealed that there were 11, 16, and 33 common pathways in CK vs. Pt2d, CK vs. Pt5d, and CK vs. Pt8d, respectively (Fig. 6). Among them, ABC transporters, cysteine and methionine metabolism, flavonoid biosynthesis, linoleic acid metabolism, pantothenate and CoA biosynthesis showed enrichment in all 3 comparisons. Compared with the untreated group, the related genes and metabolites of these 5 pathways showed differential expression and accumulation at each time point of treatment, indicating that the early response of *P. tabulaeformis* to pathogens is closely related to these functions. Compared with the untreated group, DEGs and DAMs were enriched in phenylalanine metabolism and phenylpropanoid biosynthesis during treatment for 2 and 5 days, which played a great role in the initial response. Butanoate metabolism, C5-branched dibasic acid metabolism, Lysine degradation, Purine metabolism, Tyrosine metabolism and Valine, leucine and isoleucine biosynthesis participated in late response (CK vs. Pt5d, CK vs. Pt8d). Many amino acid metabolism pathways like Beta-alanine and tryptophan metabolism (CK vs. Pt2d), Arginine and proline, Glycine, serine and threonine and Histidine metabolism (CK vs. Pt8d) were also co-enriched. In addition the energy-related pathways, such as Galactose metabolism, Starch and sucrose metabolism, Biosynthesis of unsaturated fatty acids, vitamin metabolism pathways such as Ascorbate and aldarate metabolism, Nicotinate and nicotinamide metabolism,

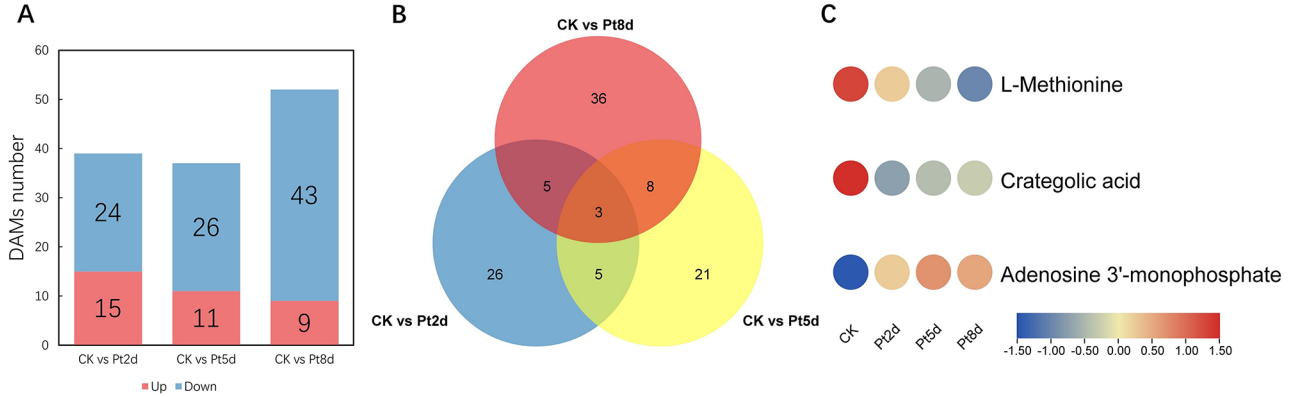

Fig. 4 Venn diagram of differentially accumulated metabolites. (**A**) DAMs in different comparisons. (**B**) Venn diagram of DAMs. (**C**) Heatmap of common DAMs at different treatment time points

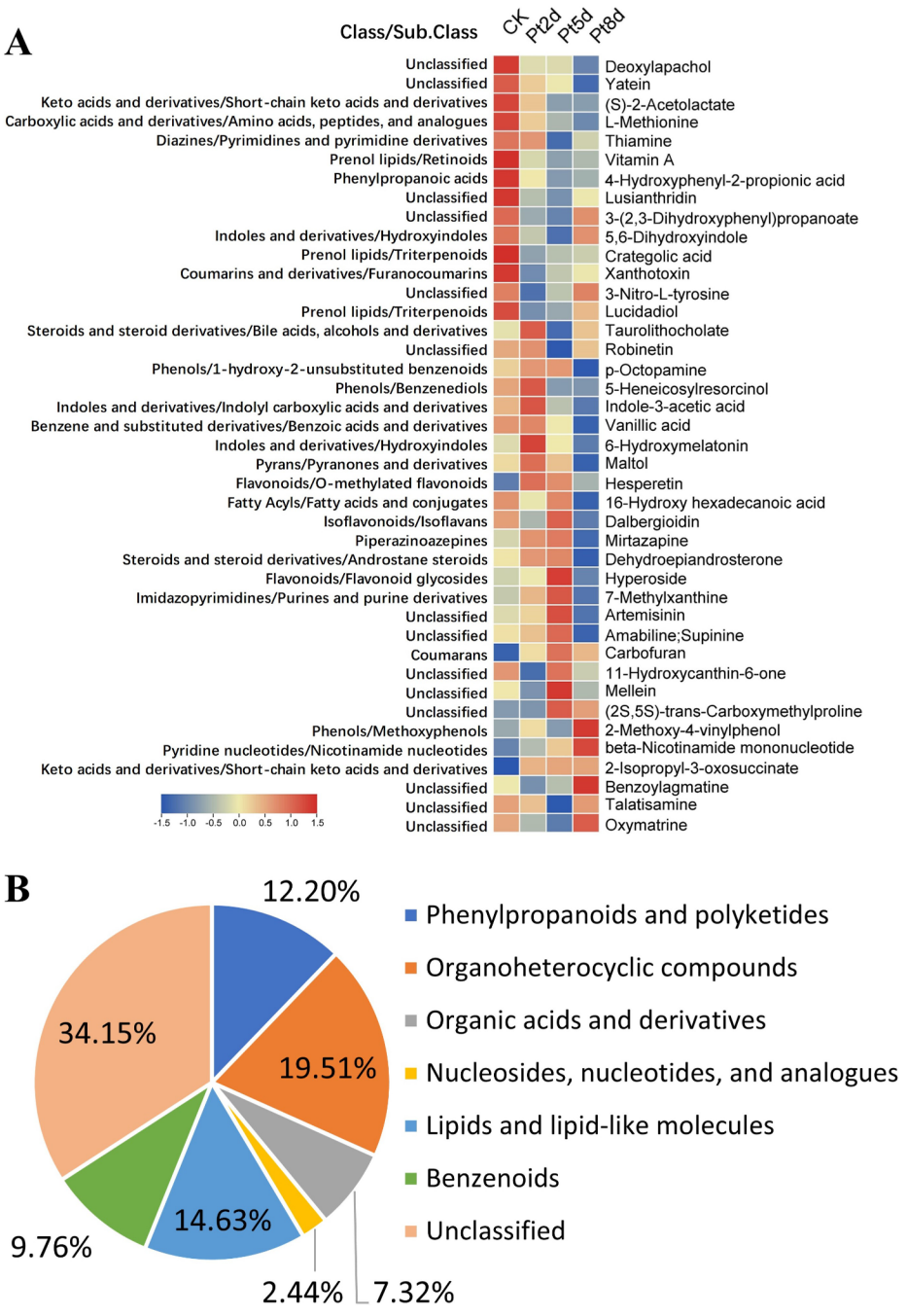

Fig. 5 Differentially accumulated metabolite analysis. (**A**) Expression of extremely significantly differentially accumulated metabolites. (**B**) Classification of highly significant differentially accumulated metabolites

and secondary metabolite-related pathways were also enriched.

Phenylpropanoid biosynthesis was found to be significantly enriched in multiple comparisons by transcriptome and metabolome analysis. Therefore, we integrated 87 DEGs and 2 DAMs in at least one comparison to map this pathway (Fig. 7). 37 *Peroxidase* (*POD*) genes were upregulated and 13 *POD* were downregulated after treatment. 3 *Cinnamyl-alcohol dehydrogenases* (*CAD*), 7 out of 10 *4-coumarate–CoA ligase* (*4CL*), 4 *Phenylalanine ammonia-lyase* (*PAL*), *Coniferyl-aldehyde dehydrogenase* (*REF*) and *Trans-cinnamate 4-monooxygenase* (*CYP73A*) were upregulated. On the contrary, 5 out of 6 *Caffeoyl-CoA O-methyltransferase* (*COMT*), 3 out of 4 *Cinnamoyl-CoA reductases* (*CCR*), 2 out of 3 *Caffeioyl-CoA O-methyltransferase* (*CCoAOMT*),

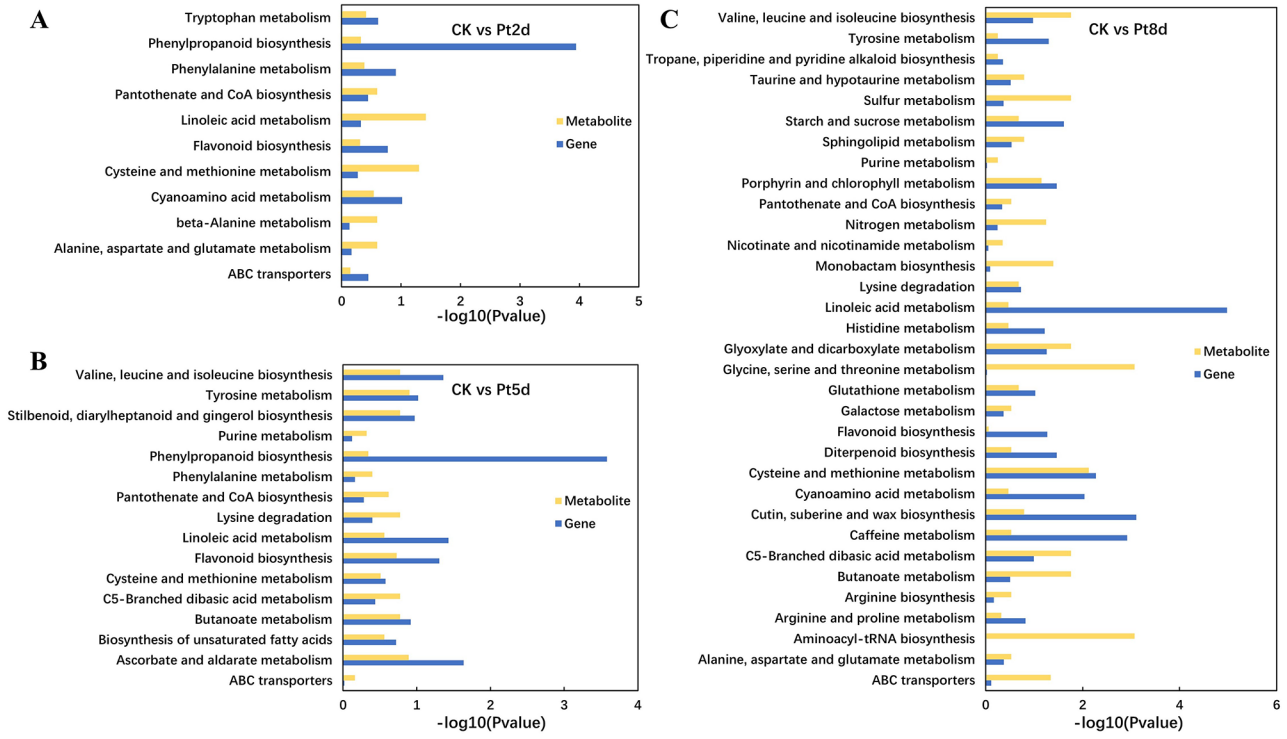

Fig. 6 Transcriptome and metabolome analysis of KEGG enrichment. (**A**) CK vs. Pt2d. (**B**) CK vs. Pt5d. (**C**) CK vs. Pt8d

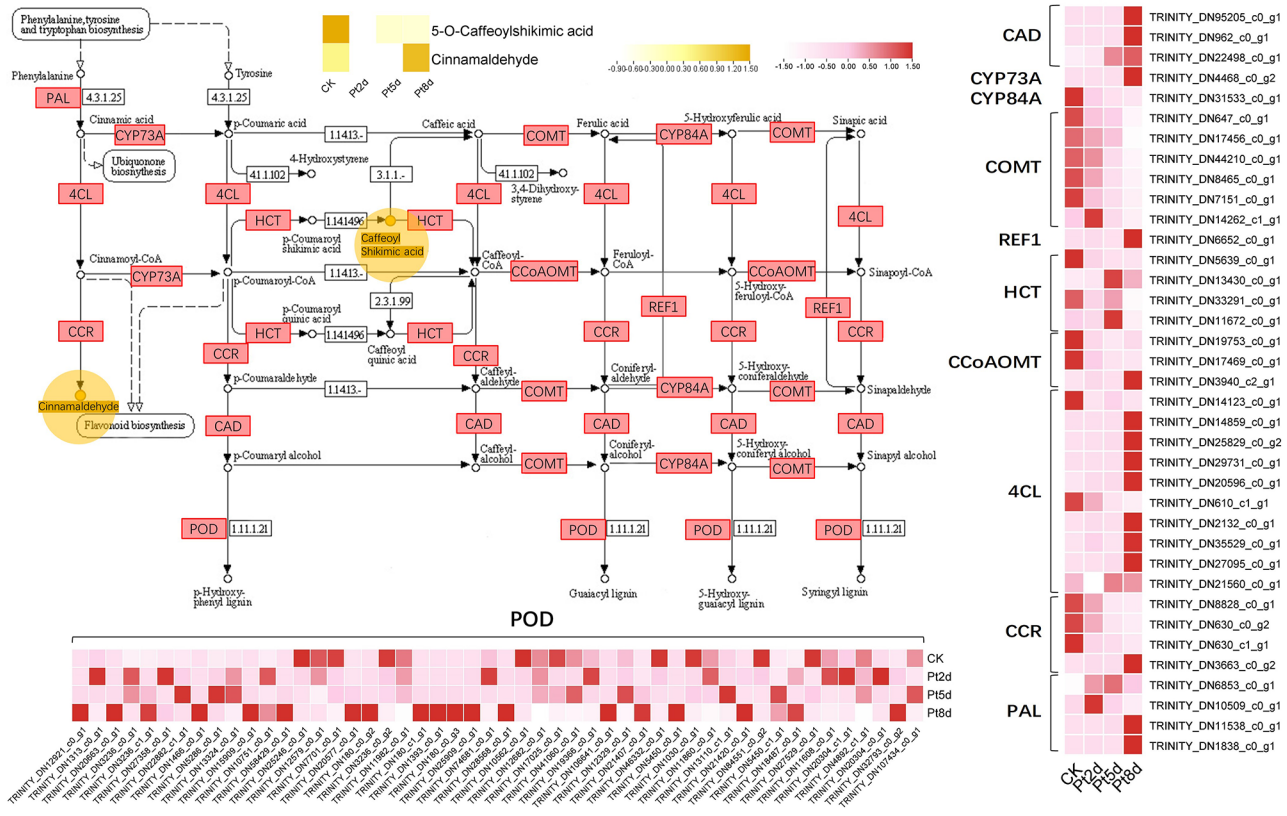

Fig. 7 Transcriptome and metabolome analysis of the phenylpropanoid biosynthesis pathway. The red box represents DEGs and the yellow circle represents DAMs

F*erulate-5-hydroxylase* (*CYP84A/F5H*), 2 out of 4 *Shikimate O-hydroxycinnamoyl transferase* (*HCT*) were downregulated. As for DAM, cinnamaldehyde was found to accumulate the highest after 8 days of treatment while 5-O-caffeoylshikimic acid decreased with treatment time. In summary, genes in the phenylpropanoid biosynthesis pathway were wildly differentially expressed, including crucial genes conserved in many plants, and affected accumulation of two metabolites.

Transcriptome and metabolome showed that flavonoid biosynthesis was significantly enriched in multiple comparisons, so we integrated 44 DEGs and 6 DAMs mapped in this pathway (Fig. 8). Among DEGs, the upregulated genes included 7 *Chalcone synthase* (*CHS*), 5 out of 6 *Flavanone 4-reductase* (*DFR*), 4 out of 6 *Anthocyanidin reductase* (*ANR*), 2 *Leucoanthocyanidin reductase* (*LAR*), *Naringenin 3-dioxygenase* (*F3H*), and *Trans-cinnamate 4-monooxygenase* (*CYP73A*). The downregulated genes included 3 *Anthocyanidin synthase* (*ANS*) and 3 *Flavonoid 3',5'-hydroxylase* (*CYP75A*). In addition, 5 *Flavonol synthase* (*FLSs*), 4 *Shikimate O-hydroxycinnamoyltransferase* (*HCTs*), 3 *Flavonoid 3'-monooxygenases* (*CYP75B1*), and 3 *Caffeoyl-CoA O-methyltransferases* (*CCoAOMTs*) were upregulated or downregulated. As for DAMs, Sakuranetin and 5-O-caffeoylshikimic acid were downregulated, while quercetin, eriodictyol, taxifolin, and hesperetin were upregulated. In summary, DEGs and

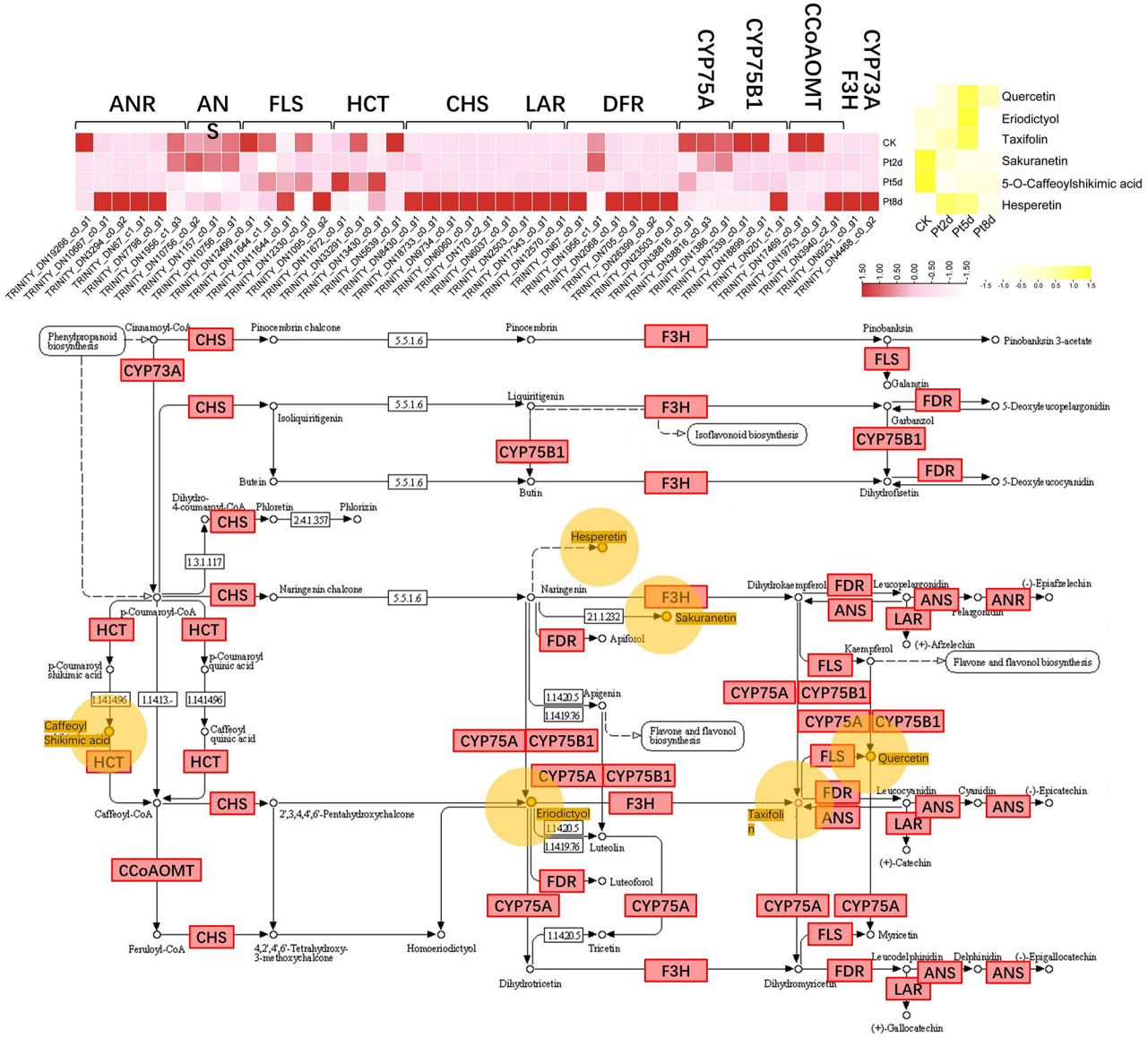

Fig. 8 Transcriptome and metabolome analysis of the flavonoid biosynthesis pathway. The red box represents differential genes and the yellow circle represents DAMs

DAMs in the flavonoid biosynthesis pathway are closely related to the pathogen response.

Discussion

Phenylpropanoid and flavonoid biosynthesis are important pathways for plants to respond to pathogens

Pine wilt disease (PWD) caused by pine wood nematodes (PWNs) is the most destructive disease of pine trees, causing worldwide economic losses. Studies have shown that host-pathogen interactions are involved in triggering phytoalexin biosynthesis after infection with PWN. Phytoalexins are defined as low-molecular-weight antimicrobial compounds produced after infection, whose accumulation is closely related to defence. Plant toxins include simple phenylpropanoid derivatives, flavonoid- and isoflavonoid-derived phytoalexins, sesquiterpenes, and polyketides [20]. At 72 h after PWN inoculation in *P. massoniana*, genes involved in secondary metabolism responded, including *CAD*, *COMT*, *CCoAOMT*, and *CHS*, which arespecifically responsible for producing secondary metabolites related to the phenylpropanoid pathway, flavonoid pathway, and lignin biosynthetic pathway [21]. After *B. xylophilus* was inoculated into *P. densiflora*, DEGs related to phenylpropanoid biosynthesis, flavonoid biosynthesis, oxidation–reduction, and plant-type hypersensitive response were significantly enriched [22, 23]. The infection of *P. massoniana* enriched phenylpropanoid metabolism and flavonoid biosynthesis, thereby reducing the levels of total phenols and total flavonoids [3]. Flavonoid biosynthesis was induced in response to late infestation (7 and 14 days post-infestation) in *P. thunbergii* Parl [24]. These studies have proven that phenylpropanoid and flavonoid biosynthesis are important pathways for plants to respond to pathogenic nematodes.

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid pathway involved in the plant defence system. PAL catalyses the first step in the phenylpropanoid pathway, converting L-phenylalanine to transcinnamic acid (CA), which is a precursor to secondary metabolites such as lignin, flavonoids, and phytoalexin. [25]. Pathogen invasion induces *PAL* expression, while the inhibition of *PAL* expression affects the mechanism of plant defence [26]. In addition, plants synthesize the important immune hormone SA salicylic acid through the PAL pathway to enhance plant disease resistance and pest resistance [27–29]. 4-Coumarate-CoA ligase (4CL) contributes to the biosynthesis of lignin and flavonoids [25]. Previous studies have shown that different plants strongly induce the expression of 4CL when infected with pathogens [30–32]. In Eastern white pine (*P. strobus*) resistant to PWNs, dihydropinosylvin monomethyl ether (DPME) and pinosylvin monomethyl ether (PME) biosynthesis involving genes such as *Phenylalanine ammonia-lyase* (*PAL*) and *4-coumarate-CoA ligase* (*4CL*) were upregulated after inoculation [33]. Multiple enzymes in the phenylpropanoid pathway are involved in lignin synthesis, including cinnamyl-alcohol dehydrogenase (CAD), caffeic acid 3-O-methyltransferase (COMT), cinnamoyl-CoA reductase (CCR), caffeoyl-CoA O-methyltransferase (CCoAOMT), ferulate-5-hydroxylase (F5H), shikimate O-hydroxycinnamoyl transferase (HCT), etc. [34, 35]. In our experiment, the expression levels of 4 *PAL*, 3 *CAD*, and 7 out of 10 *4CL* were upregulated after nematode inoculation. The results are consistent with previous studies.

One of the final products of phenylpropanoid biosynthesis is lignin, which serves as a physical barrier for pathogens. The increase in lignin content has been observed to affect resistance to nematodes in several plants [22]. The regulation of lignin components is controlled by peroxidase. Peroxidase (POD) is usually known for its role in the oxidation process, lignification, and suberization and is associated with inhibiting pathogen growth [36]. Studies have shown that maritime pine (*P. pinaster*) and Yunnan pine immediately induced *POD* family gene expression after inoculation with PWN, indicating their positive effect on PWN infection [37, 38]. In this experiment, 50 *POD* family DEGs were found, of which 37 were upregulated, indicating that *PODs* mainly responded to PWD through positive regulation in *P. tabulaeformis*. Metabolites in the phenylpropanoid biosynthesis pathway also changed significantly with nematode infestation. Cinnamaldehyde is a major bioactive compound with antibacterial, antioxidant and anti-inflammatory properties [39]. It decreased at 2 d and 5 d but accumulated to the highest level at 8 d. 5-O-Caffeoyl shikimic acid was involved in phenylpropanoid biosynthesis and flavonoid biosynthesis at the same time showing its important role, which decreased at 2 d and rebounded at 5 d and 8 d.

Flavonoids are the largest class of phenolics produced by plants as secondary metabolites to resist pathogens. Chalcone synthase (CHS) is one of the key enzymes in the flavonoid biosynthesis pathway. It also participates in the salicylic acid defence pathway, and its expression causes accumulation of flavonoid and isoflavonoid phytoalexins. In previous studies, *CHS* was highly induced (6–24 h) in the first stage after PWN inoculation in *P. pinaster* and *P. sibirica* [38, 40]. CHS catalyses the condensation of 3 molecules of malonyl-CoA and 1 molecule of 4-coumaroyl-CoA to form naringenin chalcone, which isomerizes to form various flavonoid compounds under the action of chalcone isomerase, flavonoid synthase, and isoflavone synthase [41]. *Arabidopsis* overexpressing *AeCHS* showed increased levels of flavonoids [42]. During the defence response induced by poplar leaf rust (*Melampsora medusae*) infection, upregulation of *LARs* and *ANRs* in the flavonoid pathway led to accumulation

of proanthocyanidin in *Populus trichocarpa* x *P. deltoides* [43]. In our experiment, 4 out of 7 *CHSs*, 2 *LARs*, and 4 out of 6 *ANRs* were upregulated, this conclusion is consistent with previous studies.

In addition to genes, there are significant differences in the accumulation of metabolites in the flavonoid pathway. Sakuranetin is a 7-methoxy derivative of naringenin and has been shown to have anti-inflammatory, antimutagenic and antimicrobial activities against *Helicobacter pylori*, *Leishmania* and *Trypanosoma cruzi* [44]. In rice, gene modification can be used to increase the production of naringenin and sakuranetin in a more targeted manner, reducing feeding damage by *O. hyla intricata* and *N. lugens* [45]. Sakuranetin decreased after nematode treatment in this experiment may be a characteristic of *P. tabulaeformis* injury. Quercetin [46], eriodictyol [47], taxifolin [48]and hesperetin [49]are 4 plant-active flavonoids that can slow the body's oxidative stress process by directly scavenging oxygen free radicals, chelating metal ions, inhibiting lipid peroxidation, and regulating antioxidant enzyme activity. In this experiment, their accumulation was upregulated, which may be a positive response of *P. tabulaeformis* to pathogens.

In summary, KEGG analysis in this experiment showed that DEGs and DAMs were enriched in phenylpropanoid and flavonoid biosynthesis, which are two important pathways in the early response of *P. tabulaeformis* to PWN infection.

Pinus tabulaeformis mainly responds to pine wood nematode stress by positively regulating genes

Under different treatment times, the number of upregulated genes was always higher than that of downregulated genes (Fig. 2-A). In addition, among 272 common DEGs, 26 DEGs (|Log2 (FC) | ≥ 2)shared at different treatment times were upregulated in enriched KEGG pathway (corrected p-value < 0.05, Table S1.), which may play a key role.

The SA-, ET- and JA-mediated signaling pathways are thought to be the backbone of plant immune responses against biotic invaders [50]. TRINITY_DN11212_c0_g1 encodes ETHYLENE-RESPONSE-FACTOR1 (ERF1), which regulates the expression of pathogen response genes that prevent disease progression, and acts as a key element downstream of the intersection of ethylene and jasmonate pathways [51]. ERF1 showed significant alterations between resistant and susceptible *P. thunbergii* trees [24]. Ethylene-responsive element binding proteins (EREBPs) are members of the plant transcription factor family. Based on previous research, *GmEREBP1* mRNA decreased in 'Corsoy 79' roots (susceptible) after soybean cyst nematode (*Heterodera glycines*) infection, while it increased in 'Hartwig' roots (resistant) after infection. These changes indicate that GmEREBP1 played a role in soybean cyst nematode infection. consistent with previous studies, 2 EREBP genes were upregulated after pathogen infection in our experiment [52]. TRINITY_DN865_c0_g1 encodes pathogenesis-related transcriptional activator (PTI5), which participates in ethylene-activated signal pathways (GO:0009873). *PTI5* responded to *Macrosephum euphorbiae* and was transcriptionally upregulated, contributing to plant defence by limiting the population growth of this phloem-feeding insect, and virus-induced *PTI5* gene silencing increased aphid populations on tomato [53]. The overexpression of *PTI5* in tomatoes enhanced the expression of disease-related genes and conferred resistance to the pathogen *Pseudomonas syringae* [54]. Upregulation of *PTI5* may be crucial for plants to actively defend against pathogens in this experiment. TRINITY_DN3639_c0_g1 encoding the transcription factor MYC2 was upregulated, which is involved in the response to abscisic acid (GO:0009737), response to jasmonic acid (GO:0009753), and positive regulation of the flavonoid biosynthetic process (GO:0009963). The immune hormone jasmonic acid (JA) triggers genomic transcription changes to respond to pathogen and insect attacks, while these progresses are largely regulated by the basic helix-loop-helix (bHLH) transcription factor MYC2 [55]. Transcription factor MYC2 was up-expressed in PWN-resistant *P. thunbergii* [24]. In addition, jasmonate ZIM domain proteins (JAZ) are a class of blocking proteins that participate in multiple signaling pathways. They can respond to the stimulation of JA and release the bound MYC2 transcription factor, thereby initiating the transcription of JA response genes [55].In this experiment, 4 JAZ family genes were significantly upregulated.

In addition to the ET and JA signaling pathways, TRINITY_DN3323_c0_g2 encodes an auxin responsive family gene *SAUR*. *SMALL AUXIN UP RNAs* (*SAURs*) are the largest family of early auxin responsive genes and participate in the regulation of various cellular, physiological, and developmental processes. Chitinases play a crucial role in the defence process against pathogenic microorganisms. In this study, the upregulation of 5 chitinase genes indicates the manner in which P. tabulaeformis responds to PWD. In summary, during the process of *P. tabulaeformis* responded to PWN stress, various key genes related to defence progress or transcription factors that have been widely studied were significantly upregulated in the early stage (CK vs. Pt2d) and maintained at high expression levels at subsequent times (CK vs. Pt5d, CK vs. Pt8d). These genes may be closely related to the *P. tabulaeformis* defence response and may affect or regulate the expression of downstream genes.

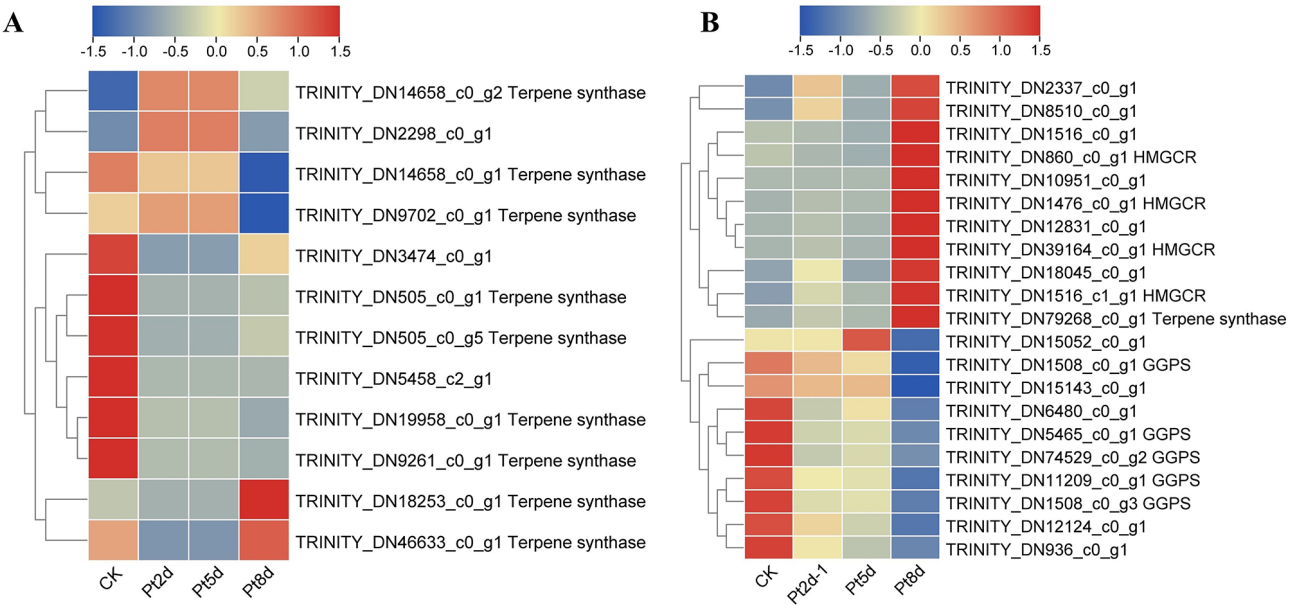

Fig. 9 Heatmap of **terpenoid biosynthesis involved DEGs.** (**A**) Diterpenoid biosynthesis pathway. (**B**) Terpenoid backbone biosynthesis pathway

The pathogenic mechanism is related to the downregulation of genes related to photosynthesis, plant-pathogen interaction, DNA replication and repair

In addition to upregulated genes, some genes showed downregulation during the treatments (Fig. 3 Clusters 2, 6, and 7), and their KEGG enrichment results showed that they are mainly involved in photosynthesis-related processes (Cluster 2) (51 genes), plant-pathogen interaction (Cluster 6) (23 genes), and DNA replication and repair (Cluster 7) (160 genes) (Table S5-7). Their downregulation may be closely related to the pathogenesis of *P. tabulaeformis.* Light-harvesting complex II (LHC II) is responsible for transferring absorbed light energy to the PSII reaction centre and maintaining the stability of the PSII electron transport chain [56]. LHC II not only participates in plant photosynthesis but also regulates plant growth and development. Silencing AtLhcb1 (light-harvesting complex II chlorophyll a/b binding protein) in *Arabidopsis thaliana* inhibited the formation of LHC II trimers, affected photosynthesis, and caused dwarfism [57]. Turnip mosaic virus (TuMV) infection downregulated the expression of *NbLHCB3* (light-harvesting chlorophyll a/b complex protein 3) in *Nicotiana benthamiana*, inducing ROS production involved in defence against TuMV [58]. Previous studies have shown that PWN-susceptible plants exhibited shutdown of central metabolism, osmolyte accumulation, photosynthetic inhibition, and a decrease in plant water status [8]. In our experiment, 9 light-harvesting complex II chlorophyll a/b binding protein genes were downregulated. In addition, 8 photosystem I subunit genes, *PsaK*, *PsaE*, *PsaF*, *PsaH*, *PsaO*, *PsaL*, *PsaN*, and *PsaG*, and photosystem II-related genes, *PsbQ*, *PsbO*, *Psb28*, *PsbP*, *Psb27*, *PsbY*, and *PsbR*, were downregulated. Glyceraldehyde-3-phosphate dehydrogenase (Gaps) catalyses key steps in energy metabolism and reduces energy distribution in cells involved in environmental stress responses [59]. In our experiment, 3 *glyceraldehyde-3-phosphate dehydrogenase (NADP+)* (GAPA) genes were downregulated. The downregulation of photosynthesis-related genes indicates damage to the reaction center in *P. tabulaeformis* during its defence process and may affect plant growth and development.

TRINITY_DN19787_c0_g1 encodes enhanced disease susceptibility 1 protein (EDS1). EDS1 participates in the resistance process against several pathogens (viral, bacterial and fungal) in various plants such as *Arabidopsis* and tomato [60–62]. TRINITY_DN9060_c0_g1 and TRINITY_DN13115_c0_g1 encode 3-ketoacyl-CoA synthase (KCS), which catalyses the biosynthesis of very long-chain fatty acid (VLCFA) wax precursors, plays an important role in wax biosynthesis and is related to stress resistance [63, 64]. TRINITY_DN12043_c0_g1 encodes LRR receptor-like serine/threonine-protein kinase flagellin-sensitive (FLS2). In *Aegilops tauschii*, transcriptional analysis revealed that FLS2 played an important role in 2 resistant ecotypes [65]. In *Arabidopsis thaliana*, FLS2 helped to resist bacterial pathogens [66]. *Disease resistance protein* 2 (*RPS2*) is a class of plant disease resistance genes rich in leucine repeat sequences that are resistant to *Pseudomonas syringae* expressing the avirulent gene *avrRpt2* [67]. In our experiment, 16 *RPS2* genes were downregulated. The downregulation of these plant-pathogen interaction genes in plants may be related to the susceptibility of *P. tabulaeformis*.

Key genes involved in DNA repair and replication included 14 genes encoding histones (histone H1/5,

histone H2A, H2B, H3, H4), TRINITY_DN20485_c0_g1 encoding chromatin assembly factor 1 subunit B (CHAF1B), and TRINITY_DN6705_c0_g1 encoding chromatin assembly factor 1 subunit A (CHAF1A). In *Arabidopsis thaliana*, chromatin assembly factor-1 (CAF-1) plays a critical role in the organization of the shoot apical meristem (SAM) and root apical meristem (RAM) during postembryonic development by facilitating stable maintenance of gene expression states [68]. TRINITY_DN15371_c0_g1 encodes BRCA1-associated RING domain protein 1 (BARD1). BARD1 has previously been implicated in DNA repair functions and regulates SAM organization and maintenance by limiting *WUSCHEL* (*WUS*) expression to the organizing centre [69]. The downregulation of these genes may indicate that plant cell proliferation, growth, and development are impaired.

Terpenoid biosynthesis genes involved during the development of pine wilt disease

The biosynthesis of resin is a significant characteristic of coniferous plants. When invaded by Pine Wood Nematode (PWN), resistant P. massoniana secretes a large amount of resin terpenoids as a defensive strategy [70]. Resin is primarily composed of terpenoids. Terpene, the simplest class of terpenoids, are typically volatile. During the development of PWD, the secondary metabolites of host may directly determine the speed of disease transmission, such as the products of the phenylpropane pathway and the isoprene-like pathways (i.e., terpene resins), which have a potent killing effect on invasive organisms [71].

There are significant differences between high and low resin-producing P. massoniana in the terpenoid backbone biosynthesis pathway (KO 00900) and the diterpenoid biosynthesis pathway (KO 00904) [72]. In this study, among the differentially expressed genes with different expression trends, Cluster 2 is enriched ($P<0.05$) in Diterpenoid biosynthesis, indicating that downregulated genes are primarily involved in this pathway. Among them, the terpene compound synthesis gene (terpene synthase, TPS) shows differential expression of 9 TPS genes in this pathway, with 7 being downregulated (Fig. 9A). Previous studies have identified two terpene synthase genes in resistant P. massoniana, *PmTPS4* and *PmTPS21*, and have demonstrated that they play an active role in the mechanism of terpene defence against PWN invasion [70]. The main products of these terpene synthases can directly inhibit the in vitro survival rate of PWN. Additionally, in this study, 4 hydroxy-methylglutaryl-CoA reductase (HMGCR) genes were upregulated and 5 geranylgeranyl diphosphate synthase (GGPS) genes were downregulated in the Terpenoid Backbone Biosynthesis pathway (Fig. 9B). In plants, HMGCR is involved in regulating the mevalonic acid (MVA) pathway, which is one of the important biosynthetic pathways for terpenoid compounds. Geranylgeranyl diphosphate synthase (GGPS) is the key synthase in terpene synthesis. Thus, a tight correlation may exist between these genes and the process of PWD. It indicates that genes in this pathway related to terpenoid biosynthesis may be regulated during the development of PWD.

Conclusions

In summary, 3-year-old *P. tabulaeformis* was sampled at different time points after infection. Through transcriptome and metabolome analysis, it was found that phenylpropanoid, terpenoid and flavonoid biosynthesis are important pathways for plants to respond to pathogens. Under PWN stress, many genes are upregulated and participate in defence, while genes related to photosynthesis, plant-pathogen interaction, and DNA replication are downregulated. The defence mechanism of *P. tabulaeformis* against PWNs was preliminarily explored.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10707-2.

Supplementary Material 1

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Author contributions

SS conceived of the work and reviewed the manuscript. Shuo Li conducted the experiments. BX processed the data and wrote the manuscript. The manuscript has been reviewed by Jinyu Qi, Liyuan Yang and Dachuan Yin. All authors read and approved the manuscript.

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Data availability

RNA-seq data is available at the SRA database in National Center of Biotechnology Information, https://dataview.ncbi.nlm.nih.gov/object/PRJNA983569?reviewer=aldmnqpr3vo3vkrnhlm1tbi31.

Declarations

Ethics approval and consent to participate

3-year-old *P. tabulaeformis* used in our research come from Chaoyang City, Liaoning Province, China. The PWN (*Bursapherenchus xylophilus*) was separated from infected *P. tabulaeformis* in Dongling Park, Shenyang City, Liaoning Province, China. The experiment was conducted in the Forest Protection Laboratory of Shenyang Agricultural University. After the experiment, harmless treatment was taken. Experimental research and field studies on plants including the collection of plant material comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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