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DE NOVO ASSEMBLY AND DISCOVERY OF METABOLIC PATHWAYS AND GENES THAT ARE INVOLVED IN DEFENSE AGAINST PESTS IN SONGYUN *PINUS MASSONIANA* LAMB.

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Abstract

Pest-resistant strains of *Pinus massoniana* Lamb. can defend themselves against pests. However, the mechanisms underlying pest resistance in them remain. Next-generation sequencing (NGS) technology was employed to examine the transcriptomes of insect-resistant and insect-sensitive strains of *P. massoniana*. In this investigation 98,547 transcripts and 55,824 unigenes were obtained. Among the unigenes, 35,214 sequences (63.08%) were annotated. Gene expression levels in strains with various levels of insect resistance were compared. After this comparison 511 genes were found to be differentially expressed. Pathway analysis of the differentially expressed genes revealed a number of important pathways, including the "terpenoid metabolic pathway" and the "flavonoid metabolic pathway". The present study revealed that several important transcription factors (TFs), including WRKY and the Apetala2/ethylene responsive factor (AP2/ERF), as well as important enzymes such as abietadiene synthase, play significant roles in anti-pest responses. Discovery of these defense genes and pathways is conducive to further understanding of the mechanisms by which *P. massoniana* defends itself against pests. In addition, 11 genes that were assumed to participate in anti-pest responses were examined by real-time quantitative PCR to verify the accuracy of the transcriptome data. This study identified the metabolic pathways and related genes expressed by insect-resistant *P. massoniana* varieties to improve their ability to defend against pests. The above data will provide multidimensional information regarding the molecular mechanisms of insect resistance in PM for the future researchers.

Introduction

Pests are an important factor affecting agricultural production and can cause serious economic and ecological losses. Long-term heavy application of pesticides not only induces pesticide resistance but also causes a series of social and environmental problems (Jander 2012, He *et al.* 2012). Cultivation of pest-resistant species is the most cost-effective way to solve pest problems. With the advances in molecular biology and bioinformatics, in-depth study of the mechanisms of insect resistance and mining of relevant insect-resistance genes have become hotspots in plant genetic engineering research and its practical application. In recent decades, a large number of studies on the mechanisms underlying the constitutive and induced plant resistance against pests have been conducted (Jander 2012, Liu *et al.* 2012). Novel potential biochemical pathways are constantly being discovered, and our understanding of the mechanisms driving insect resistance is continuously improving, opening new directions for revealing the mechanisms underlying insect resistance in plants. All plants have their own characteristic scents. Certain substances stimulate plants to exude these odors to reject insects from feeding on them (Xin *et al.* 2012). In addition,

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various secondary metabolites produced by plants have decisive influences on insect feeding behavior. Many of these secondary metabolites (i.e., secondary plant substances) are capable of providing a means of defense against insect feeding or poisoning the insects. Currently, research on the genes that encode the key enzymes in these secondary metabolic pathways is still in the primary stage.

Pinus massoniana is a native coniferous species that is typically grown in southern China. It accounts for 16% of the forest volume in South China, and produces more than half of the world's turpentine (Zhao and Jiang 2014, Fan *et al.* 2014, Wang *et al.* 2015).The pine caterpillar (*Dendrolimus punctatus* Walker) and the pine tussock moth (*Dasychira axutha* Collenette) are the pests that cause a significant reduction in the production of pine-related forest products, thereby restricting the development of the pine industry. To date, however, the molecular mechanisms by which *P. massoniana* defends itself against pests have not been described.

The *P. massoniana* variety "Songyun", which is highly resistant to the pine caterpillar and the pine tussock moth, was used as the research material. The Songyun variety was developed by our research group through 10 years of selective breeding. In addition, the most advanced transcriptome sequencing technique was employed in this study. The aims of this study were to thoroughly understand the mechanisms underlying pest resistance of *P. massoniana*, to discover potential pest-resistance substances and resistance genes in it and to provide a theoretical basis for achieving sustainable control of pest damage.

Materials and Methods

The insect-resistant *P. massoniana* variety Songyun was audited by the office for the protection of new varieties of plants of the State Forestry Administration of the People's Republic of China. The control (CK) was a *P. massoniana* variety with poor insect resistance, "GC105", which was selected after analysis of insect resistance. The materials were cultivated at the *P. massoniana* Germplasm Resources Garden of the Guangxi Academy of Forestry (Nanning, Guangxi Province, China) and were collected in August 2014. Tender leaves, tender stems, old leaves and old stems were collected from similar sites of "Songyun" and "GC105"at the same time. The materials were placed in liquid nitrogen immediately after collection and then stored in an –80°C freezer prior to use in assays.

RNAs were extracted from all materials using a commercially available RNA extraction kit for polyphenol- and polysaccharide-rich plants (Tiangen Biochemical Technology Co., Ltd., Beijing, China). Subsequently, the mRNAs were isolated and purified according to the instructions provided with the Dynabeads mRNA Purification Kit (Invitrogen, Carlsbad, CA, USA). The enriched mRNAs were broken into short segments by the addition of fragmentation buffer. Single-stranded complementary DNAs (cDNAs) were synthesized using the mRNAs as templates and random hexamers as primers. Corresponding double-stranded cDNAs were then synthesized. After purification, the double-stranded cDNAs underwent end repair, addition of A-tails, ligation of sequencing adapters, and fragment size selection using AMPure XP beads. Finally, PCR amplification was performed. The PCR products were purified using AMPure XP beads to establish the libraries. The libraries were quantified using the Qubit® RNA Assay Kit on Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) and then using a Nano 6000 Assay Kit ona Bioanalyzer 2100 system (Agilent Technologies, Santa Rosa, CA, USA). The two sequencing libraries constructed in the present study were sequenced simultaneously on the Illumina HiSeq2000 high-throughput sequencing system using the paired-end sequencing method by Novo gene Bioinformatics Technology Co. Ltd. (Beijing, China; www.novogene.cn). The small-fragment library consisted of fragments 300 bp in length. The sequence data were assembled using

Trinity software (version: v2012-10-05, parameter settings: min_kmer_cov was set to 2, other parameters used the default values) (Grabherr *et al.* 2011).

The obtained unigenes and transcript sequences were subjected to functional annotation, functional classification, differential gene expression analysis and metabolic pathway analysis using the following seven functional databases: The National Center for Biotechnology Information (NCBI), non-redundant protein sequence (NR) database, the NCBI nucleotide sequence (NT) database, the protein family (PFAM) database, the Eukaryotic Orthologous Groups (KOG) database, the Swiss-Prot protein database, the Gene Ontology (GO) database and the KEGG Ortholog (KO) database (Kanehisa 2008). To further analyze the expression levels of the genes, the read counts were converted into FPKM (expected number of fragments per kilobase of transcript sequence per million base pairs sequenced). In RNA sequencing (RNA-seq) technology, FPKM takes into account the effects of sequencing depth and gene length on the fragment counts and is the method most commonly used to estimate gene expression levels (Trapnell *et al.* 2010). Adjusted p-values of 0.05 and log2 (fold change) greater than 1 was set as the threshold for significant differential expression.

A total of 11 genes were selected from various metabolic pathways containing differentially expressed genes. Primers for fluorescence-based quantitative PCR were designed accordingly. Based on the reference gene screening results obtained previously (Chen *et al.* 2016), the polyubiquitin (*UBI4*) gene and the cytochrome P450 (*CYP*) gene were selected as the reference genes (Table 1). The cDNAs were synthesized from the extracted RNAs using an oligo $(dT)_{18}$ reverse transcription primer in accordance with the instruction manual of the M-MLV reverse transcriptase. The fluorescence-based quantitative PCR system and the amplification program were set up according to the instruction manual of the SYBR Premix Ex Taq II (Prefect real-time) Kit (Takara Biotechnology Co., Ltd.). PCR amplification was conducted on the Light Cycle 480II Real-Time PCR instrument. All experiments were repeated three times. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and plotted using Excel.

Table 1. The sequence of primer for qRT-PCR.

	Forward primer	Reverse primer
UBI4	AGCTCCGACACCATTGATAA	CCAAAGTACGTCCATCTTCCA
CYP	CAAGGGTTCGTCGTTCCAC	GGCAAACTTCTCGCCGTA
comp48665_c0	CCCAAGGTGGCTTACAATCA	TGCTCACTTCGTCTCTCACG
comp48116_c0	TGGCGGAAGTAATCAACAAC	CCACAAAGTAGGGCAGGTTC
comp47877_c0	TACATTGGACCTGTGGATGG	CCCTTTCCTTTCTCTGTCACC
comp49774_c0	TGGGAGAAACATCTGGGCTA	AGCAGGACGTGGAGACAACT
comp18274_c0	GCCCGCTTCCAGAGATTAC	TTGGGTTTTGACCTTTAGCA
comp32160_c0	TCAAACTCCCCGTCTTATGG	GCTCCAACTGAATCCTTGC
comp50694_c0	AATCGTTGTTCACACGTTGC	GTCAAAGGCATCCACATCCT
comp42878_c0	ACGGTGCCCTTCTTTACCA	CCTCGGGTCGTTTCTTGAT
comp41737_c0	GGGACGCTGCATATCAATCT	ACGGTGAACGCAAACTTCTC
comp37634_c0	TACGCACGCCATTACGACTA	TGGAACAACACCTCCTGCTA
comp35916_c0	ACTGGATGGTTCCTCAAACG	AACAGATTGCTCCCTGGTTG

Results and Discussion

Pests are a major factor that severely restricts the production of *Pinus massoniana*. Investigations of *P. massoniana* related mechanisms from the perspective of molecular biology are rather rare, which has seriously hampered the breeding process of *P. massoniana*. To date, no study has been focused on the mechanism(s) by which *P. massoniana* defends itself against pests.

A total of 98,547 transcripts and 55,824 unigenes were obtained after *de novo* assembly using Trinity (Grabherr *et al.* 2011). Unigenes of 200 - 500 bp in length were accounted for the largest proportion (57.70%) of the unigenes obtained. Unigenes with lengths of 0.5 - 1 kbp and 1 - 2 kbp accounted for 16.27 and 15.23% of all unigenes, respectively. The total length of the 72,224 unigenes was approximately 49.5 Mb. The average length of the unigenes was 863 bp, and the N50 length of the unigenes was 1696. The above values are significantly larger than the comparable values for the single library, demonstrating the high assembly efficiency of Trinity software in the absence of a reference genome and the reliability of Illumina paired-end sequencing technology (Yang *et al*. 2013, Li *et al.* 2015, Zhang *et al.* 2015).

The assembled unigene sequences were subjected to sequence similarity search against the listed seven public data bases. The results show that a total of 55,824 unigenes were successfully annotated. Among the annotated unigenes, 35,214 unigenes (63.08%) were annotated by at least one database, whereas 4,741 unigenes (8.49%) were annotated in all seven databases.

In the GO analysis, 23,961 unigenes were divided into three main GO categories (Biological process (BP), cellular component (CC) and molecular function (MF)) and then further divided into 47 subcategories (Fig. 1). The largest proportion of the genes was involved in cellular processes, metabolic processes, binding processes and catalytic activity.

All unigenes were aligned to the COG database for functional prediction and classification. The results showed that 11,286 unigenes were annotated and that these were divided into 26 specific categories. All genes were divided into further divided into 32 sub-branches in KEGG. The largest number of the genes was involved in metabolic pathways.

The gene expression level of the unigenes was quantified using FPKM. In the present study, a total of 511 genes were found to be significantly differentially expressed. Of the 511 genes, 327 were up regulated and 184 were down regulated. The number of differentially expressed genes identified in the present study is low compared to the number of differentially expressed genes that have been found in most other experimental conditions (Qi *et al.* 2014). In addition, 47 genes were novel genes, accounting for 9.2% of the differentially expressed genes. A total of 154 genes (30.1%) were successfully annotated in the NR database. Of the 511 differentially expressed genes, 59 were important enzyme genes, and 41 of these genes were up regulated in the insect-resistant strains. In addition, the 511 differentially expressed genes included eight genes of the P450 family, 34 genes encoding ATP binding proteins and four genes encoding heat shock proteins. These results indicate that the strains selected for the present study share a highly similar genetic background, which ensured the reliability of our data. In addition to identifying differentially expressed genes, we analyzed the expression of 11 genes that are involved in various metabolic pathways in the same strains and thereby further varified the reliability of our results.

Plants employ complex gene regulatory networks to defend themselves against pests. TFs have been shown to play important roles in the regulation of anti-insect responses (Jander 2012). Of the 511 genes differentially expressed between the two strains with different levels of insect resistance, 67 were identified as TF genes. These 67 genes were classified into 14 categories. The zinc finger, ABC and Apetala 2/ethylene responsive factor (AP2/ERF) TF families were the three largest TF families which has been shown to respond to various abiotic and biotic stresses (Bethke *et al.* 2009), containing 59.71% of all differentially expressed TFs (Table 2). To date, there are

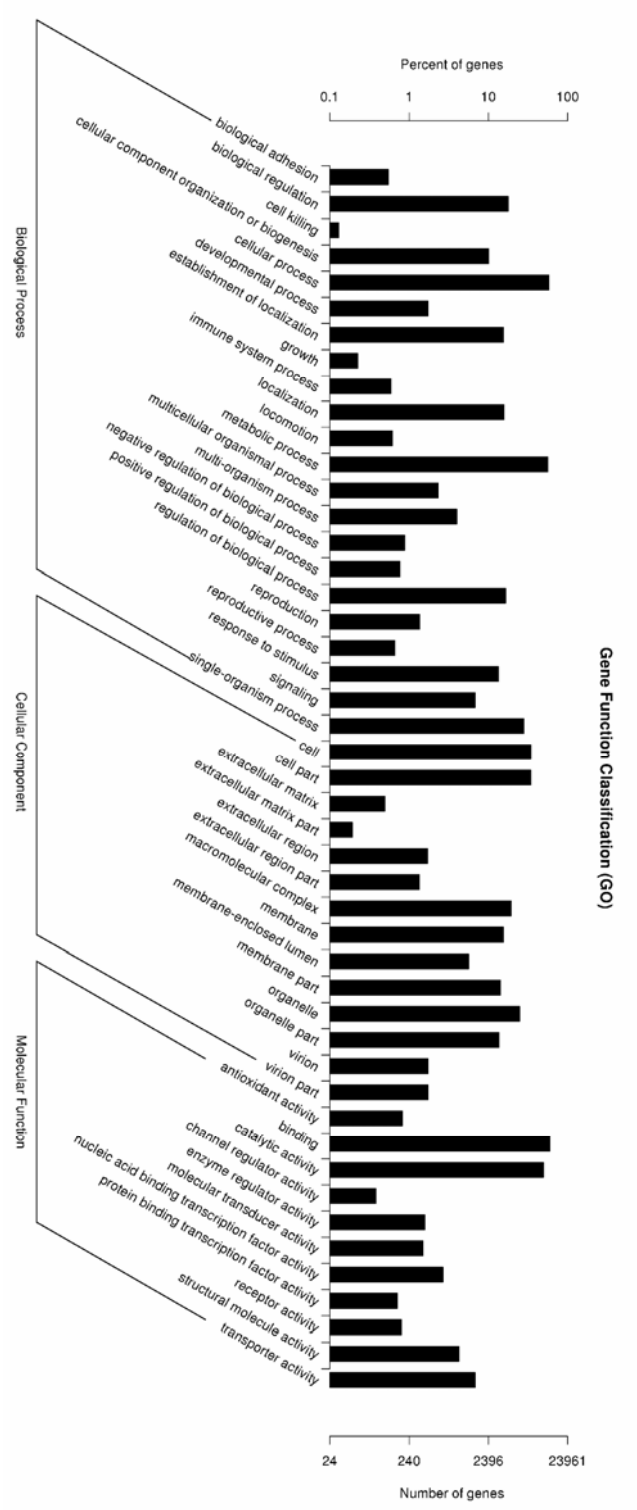

Fig. 1. GO classification of unigenes of *Pinus massoniana* Lamb. The results are summarized in three main GO categories: Biological process, cellular component and molecular function.

reports on the biological functions of ABC, zinc finger, MADS or NAC TFs in plant defense responses to pests. However, numerous studies show that those TFs are involved in plant responses to biotic and abiotic stresses. In addition, WRKY, NAM/ATAF1/2/CUC2 (NAC), MYB and mini chromosome maintenance 1-agamous-deficiens-serum response factor (MADS) TFs were also found. WRKY have long been known to participate in anti-insect pathways in tobacco (Jiang *et al.* 2014), whereas MYB TF has also been proven to confer increased pest resistance (Kaur *et al*. 2010). Of the differentially expressed TF genes, 43(64.17%) were up regulated in insect-resistant material and 14 were down regulated. The WRKY, AP2/ERF and TCH TFs were all up regulated (Table 2). The present study identified 76 WRKY family members among the annotated sequences. Of these, only WRKY1 (comp50882_c0), WRKY108 (comp46495_c0) and WRKY6 (comp21859_c0) showed significant changes in gene expression levels, a finding that is consistent with the results of a study conducted on rice (Hu *et al.* 2012).

Table 2. Regulated transcription factors during in insect resistance.

Gene family		WRKY	MYB	Zinc finger	MADS	AP2/ERF	ABC	NAC	Sugar trans-porter	TCP	bHLH	Other	Total
No.		3	2	15	3	11	14	2	9	1	1	6	67
Percentage		4.48	2.99	22.39	4.48	16.42	20.90	2.99	13.43	1.49	1.49	8.96	100.0
Ir vs CK	Up	3	1	11	1	11	11	1	8	1	-	5	43
	Down	-	1	4	2	-	3	1	1	-	1	1	14

Pathway-based enrichment analysis is conducive to further understanding the biological functions of the genes. A total of 71 significantly enriched pathways were identified, and 255 differentially expressed genes were annotated in the KEGG pathway analysis. Five of the differentially expressed genes identified in the present study, comp48665_c0, comp47911_c0, comp42878_c0, comp48116_c0 and comp47411_c1, are involved in the flavonoid biosynthesis pathway. Two of the differentially expressed genes were found to be in the diterpenoid biosynthesis pathway. Flavanone 3-hydroxylase (F3H) is a key upstream enzyme in the flavonoid biosynthetic pathway. Up regulation of the expression of F3H promotes the enzymatic activity of flavonoid 3',5'-hydroxylase (F3'5'H) and enhances the levels of several secondary metabolites such as dihydrotricetin, dihydromyricetin, myricetin, quercetin and eriodictyol, thereby improving the pest resistance of *P. massoniana* (Wang *et al.* 2014). In addition, the linoleic acid metabolic pathway, the thiamine metabolic pathway and the arachidonic acid metabolic pathway were among the main pathways contributing to increased insect resistance.

Numerous studies have demonstrated that treatment with hormones such as jasmonic acid, gibberellin and ethylene significantly increases the terpenoid content of plants, thereby enhancing the plant's resistance to pests (Schenk *et al.* 2014). Of the differentially expressed genes identified in this study, 59 were enzyme genes and 41 displayed upregulated expression. The abietadiene synthase (AS) gene encodes the key protein in diterpene biosynthesis. AS can catalyze formation of terpenoids from geranylgeranyl diphosphate through a series of reactions (Anisimova *et al.* 2011). Most secondary metabolites produced by plants increase the plants' resistance to living organisms that harm plants, especially terpenoids, alkaloids, flavonoids, quinones, phenols, tannins and sterols (Abbott *et al.* 2010). It is certain that genes involved in various secondary metabolic pathways whose expression is up regulated in the insect-resistant strains examined in the present study play important roles in enhancing the content of these secondary metabolites in their respective metabolic pathways. This conclusion is confirmed by the analysis conducted by

our research group on the volatile secondary metabolites terpenoids. The content of 60 individual terpenoids was increased in insect-resistant strains. Moreover, the content of five terpenoids was 8-10 times higher in the insect-resistant strains than in the controls, and three terpenoids were absent in the controls. The differentially expressed genes also included eight sugar transport proteins and 34 ATP-binding proteins.

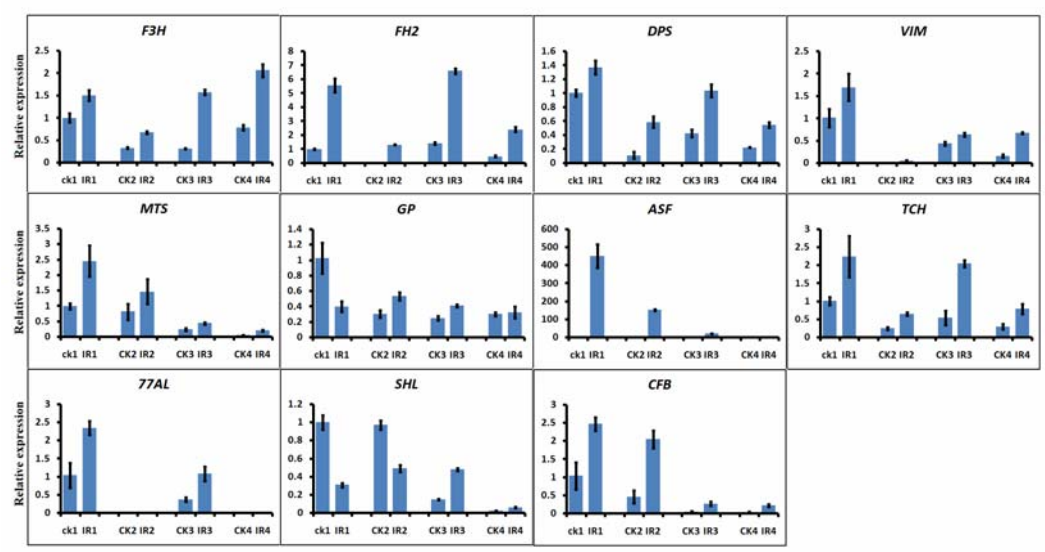

Fig. 2. Real-time PCR validations of 11 candidate DEGs in different insect resistance material. The Y-axis represents the relative expression, and the X-axis depicts the different insect resistance material and tissues. The standard error of the mean for three biological replicates is represented by the error bars. 1-4 stand for samples of tender leaf, older leaf, tender stem, older stem, respectively.

To verify the reliability of the RNA-Seq data, 11 unigenes were selected in various metabolic pathways, including flavonoid biosynthesis (comp48665_c0), thiamine metabolism (comp47877_c0), selenocompound metabolism (comp49774_c0), and arachidonic acid metabolism (comp32160_c0).The transcription levels of the 11 unigenes were analyzed using real-time quantitative PCR. As shown in Fig. 2, the expression levels of the 11 genes, including expression in the needles and stems of the plants, were higher in insect-resistant material than in control material (except for the expression of comp37634_c0 and comp32160_c0 in the needles). In summary, all the unigenes displayed consistent expression patterns. The PCR results are consistent with the RNA sequencing data. PM is a gymnosperm and possesses a unique turpentine biosynthesis system. Whether the TFs identified in the present study enhance the ability of *P. massoniana* to resist pests through the regulation of downstream genes and through participation in hormonal signaling will be the focus of our future study.

Data archiving statement of high-throughput sequencing and raw data are available at GEO database accession number GSE72294.

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