Transcriptomic and Metabolomic Analyses to Unravel Responses of *Populus Simonii* × *P. Nigra* to attack by Asian Gypsy moth *Lymantria Dispar* (Lepidoptera: Lymantriidae)

Yuntong LV

Northeast Forestry University

Lili Sun

Northeast Forestry University

Jingjing Yin

Northeast Forestry University

Yanpeng Jia

Northeast Forestry University

Hui Du

Northeast Forestry University

chuanwang cao (✉ chuanwangcao@nefu.edu.cn)

Northeast Forestry University School of Forestry <https://orcid.org/0000-0002-2674-7714>

Research article

Keywords: *Populus simonii* × *P. nigra*, transcriptome, metabolome, *Lymantria dispar* herbivory, induced resistance

Posted Date: October 9th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-70681/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Poplar is frequently attacked by herbivorous insects, including the Asian gypsy moth, *Lymantria dispar*. Here, we combined metabolomic and transcriptomic analysis to identify key genes and metabolites involved into the molecular mechanism of defensive enhancement against *L. dispar* herbivory on poplar.

Results: The 3666 differentially expressed genes (DEGs, 1,799 up-regulated and 1,867 down-regulated) and 1,171 DEGs (695 up-regulated and 476 down-regulated) were specific in *L. dispar* herbivory and mechanical wounding, respectively. Moreover, the 9,108 and 7,656 ions were detected while 636 and 531 different ions were obtained using positive (pos) mode and negative (neg) mode, respectively. Among these ions, the 33 and 7 different ions were specific in *L. dispar* herbivory and mechanical wounding in pos mode while 46 and 4 different ions in *L. dispar* herbivory and mechanical wounding in neg mode, respectively. The 3,666 specific DEGs in *L. dispar* herbivory group were classified into phenylpropanoid and flavonoid secondary metabolism pathways by comprehensive networks between transcriptomes and metabolomes.

Conclusions: The current findings greatly improve our understanding of the induced defensive response in poplar plants against *L. dispar* infestation, and will contribute to develop insect-resistance poplar varieties.

Background

Plants are sessile organisms and continuously challenged by abiotic and biotic stress factors in their natural habitat. In co-evolution, plants have developed specific mechanisms to precisely detect environmental changes and respond to complex abiotic and biotic stress conditions to minimize damage and conserve sufficient resources for growth and reproduction [1]. Insect herbivory is a major biotic stress in the natural ecosystem. However, plants have evolved sophisticated inducible and constitutive defenses against the effects of insect attacks [2]. Inducible defenses often act as toxins, antifeedants, or antinutrients. For example, many plants synthesize proteinase inhibitors induced by herbivores, which inhibit insect digestive enzymes and produce directly toxic effects [3]. A thousand of known chemical structures are produced in phenylpropanoid pathway, including simple phenolics, lignans, coumarins, flavonoids, stilbenes, and the structural polymer, lignin [4, 5]. In poplar defense, plant phenolics, including the phenolic glycosides, hydroxycinnamate derivatives, and condensed tannins, play known or putative roles [6].

Populus simonii × *P. nigra* is an improved clone that was selected from hybrid clones of *P. simonii* and *P. nigra* in the 1970s [7]. As a rapidly growing species, *P. simonii* × *P. nigra* widely distributed in North China, which can adapt to a certain degree of low temperature. Due to its wide adaptability and high tolerance to abiotic stress, the poplars have been planted in commercial forests and landscape enhancement among North China. Just like other poplar species, *P. simonii* × *P. nigra* is continuously exposed to biotic and

abiotic invasions [8]. Asian gypsy moth, *Lymantria dispar*, is a devastating chewing pest in forestry [9]. Among integrated pest managements, the potential mechanisms of plant-insect interactions and the identification of the new strategy to improve plant resistance have been considered as a perspective control strategy.

Nowadays, high-throughput omics techniques, including genomic, transcriptomic, proteomic, metabolomic, phenomic, ionomic, have been extensively employed into plant-insect interactions [10]. To further understanding specific defense of *P. simonii* × *P. nigra* against *L. dispar* herbivory, we conducted a combined transcriptomic and metabolomics analysis to excavate differences in transcript and metabolic levels of poplar leaves under *L. dispar* herbivory and mechanical wounding treatments. The resulting data will promote to better understand the underlying molecular defensive mechanisms of poplar and explore more new biological control strategy against forest insect pests.

Results

Transcriptome assembly

To investigate the response of poplar to *L. dispar* herbivory and mechanical wounding at the transcriptome level, the output of high-quality clean reads was obtained from paired-end BGISEQ-500 RNA-Seq (Supplementary Table S1). The clean reads generated from 68.94 to 69.78 Mb among all treatments. Among them, 98.11 ~ 98.36% of bases had a Q ≥ 20, whereas 90.91 ~ 91.86% of bases a Q ≥ 30. The total length, average length, N50 and GC content of unigenes were 109.9 Mb, 1,567 bp, 2,222 bp and 41.68%, respectively. All unigenes was annotated into six functional databases, and 62,326 (88.89%), 47,802 (68.18%), 49,093 (70.02%), 47,332 (67.51%), 48,222 (68.77%) and 48,414 (69.05%) unigenes were grouped into the Nr, GO, KOG, SwissProt, KEGG and Pfam databases, respectively (Table 1). The annotated unigenes were grouped into 25 KOG categories (Supplementary Fig. S1), 42 GO subgroups in three main groups (Supplementary Fig. S2), and 20 KEGG subgroups in five main groups (Supplementary Fig. S3).

Table 1
Annotation of Nr unigenes against public databases

Database	Number of unigenes	Percentage (%)
Nr	62,326	88.89%
GO	47,802	68.18%
KOG	49,093	70.02%
SwissProt	47,332	67.51%
KEGG	48,222	68.77%
Pfam	48,414	69.05%
Annotated in all databases	29,507	42.08%
Annotated in at least one database	66,779	95.24%
Total unigenes	70,116	100%

Differential transcriptome reconfiguration by *L. dispar* herbivory and mechanical wounding

By the comparative transcriptome analysis, a total of 12,152 DEGs (6,733 up-regulated and 5,419 down-regulated genes) were identified between the *L. dispar* herbivory group and the control group, and 6,440 DEGs (3,998 up-regulated and 2,442 down-regulated genes) were found between the mechanical wounding group and the control group. Moreover, the 3,790 up-regulated genes and 4,145 down-regulated genes were identified between *L. dispar* herbivory and mechanical wounding group (Fig. 2). The venn diagram indicated 3,666 DEGs (1,799 up-regulated genes and 1,867 down-regulated genes) and 1,171 (695 up-regulated genes and 476 down-regulated genes) were specific in *L. dispar* herbivory and mechanical wounding, respectively (Fig. 3).

According to the KEGG annotations, the 3,666 DEGs in *L. dispar* herbivory group and 1,171 DEGs in mechanical wounding group were involved into five categories of “metabolism”, “genetic information processing”, “environmental information processing”, “organismal systems” and “cellular processes”. In metabolism category, more systemic DEGs in *L. dispar* herbivory group and mechanical wounding group were classified into “global and overview maps” (744 genes and 198 genes), “carbohydrate metabolism” (312 genes and 81 genes) and “energy metabolism” (199 genes and 19 genes), respectively. In genetic information processing, the DEGs in *L. dispar* herbivory group and mechanical wounding group were group into subcategory “translation” (169 genes and 41 genes) and “folding, sorting and degradation” (124 genes and 46 genes), respectively. In “environmental information processing” and “organismal systems”, the DEGs in *L. dispar* herbivory group and mechanical wounding group were involved into “signal transduction” (175 genes and 57 genes) and “environmental adaptation” (105 genes and 45 genes), respectively (Fig. 4). These results indicated that herbivory triggered a large scale of transcription

reconfiguration, and the induced molecular mechanism of the herbivory response was different from those of the mechanical wounding responses.

Non-targeted metabolic profiles of poplar leaves associated with *L. dispar* herbivory

To overview the metabolic shifts over the process in response to herbivory and wounding, non-targeted metabolic profiling was performed using liquid chromatography-mass spectrometry (LC-MS). Nine samples (three control, three *L. dispar* herbivory and three mechanical wounding) were analyzed after 24 h treatment. The differential metabolites by fold change ≥ 1.2 or ≤ 0.8333 and p -value ≤ 0.05 were obtained in pos mode and neg mode, respectively (Fig. 5). Totally, 9,108 and 7,656 ions were detected while 636 and 531 different ions were obtained using positive (pos) mode and negative (neg) mode, respectively. The venn diagram indicated 33 and 7 different ions were specific in *L. dispar* herbivory and mechanical wounding in pos mode while 46 and 4 different ions in *L. dispar* herbivory and mechanical wounding in neg mode, respectively (Fig. 5).

Differential metabolite profiling in poplar leaves responding to herbivory and mechanical wounding

To understand the difference of metabolic levels between herbivory and mechanical wounding, metabolite profiles were analyzed between the CK-vs-GH, CK-vs-M and M-vs-GH by the LC-MS. Principal component analysis (PCA) showed that metabolomes of poplar leaves were separated under herbivory and mechanical wounding conditions compared with the control condition by the PC2 (Fig. 6). Under herbivore condition, the PC2 could separate the difference between *L. dispar* herbivory and control groups, which explains 28.51% of the total metabolome variation among samples in neg mode, and 26.91% of the total metabolome variation in pos mode. The dominated components for the PC2 were pantothenol, 4'-cinnamoylmussatioside and others in neg mode, and vinblastine sulfate, coenzyme B and others in pos mode, correspondingly, ubiquinone-6 (37.62-fold) and cimicifugoside (53.89-fold) were obviously up-accumulated in neg mode and pos mode, respectively (Fig. 6A and Fig. 6B).

Under mechanical wounding condition, the PC2 could separate the difference between mechanical wounding and control groups, which explains 19.85% of the total metabolome variation of among samples in neg mode, and 20.54% of the total metabolome variation in pos mode. The dominated components for the PC2 were phylloquinone, eriojaposide B and others in neg mode, and 1D-1-Guanidino-1-deoxy-3-dehydro-scylo-inositol, strictosamide and others in pos mode, correspondingly, arbutin (59.91-fold) and strictosamide; (2.17-fold) were obviously up-accumulated in neg mode and pos mode, respectively (Fig. 6C and Fig. 6D).

Comprehensive Networks Of Transcriptomes And Metabolomes

To explore the specific transcriptional characterization of the herbivory responses, 3,666 specific DEGs in *L. dispar* herbivory group were classified into secondary metabolism pathways according to KEGG

pathway analysis, which showed a large amount of DEGs involved into the phenylpropanoid and flavonoid biosynthesis (Fig. 7 and Supplementary Table S2). Thirty eight and thirty DEGs were involved into phenylpropanoid biosynthesis pathway and flavonoid biosynthesis pathway, respectively.

In the phenylpropanoid biosynthesis pathway, a phenylalanine ammonia-lyase (PAL) gene was up-regulated in the *L. dispar* herbivory group and down-regulated in the mechanical wounding group compared with control group. Of six 4-coumarate-CoA ligase (4CL) genes, all 4CL genes were significantly induced in the *L. dispar* herbivory group compared with the mechanical wounding group. Of ten cinnamoyl-CoA reductase (CCR) genes, expression levels of six CCR genes in the *L. dispar* herbivory group were 2.31 ~ 12.89-fold of those in the mechanical wounding group. Compared with the mechanical wounding group, three of four cinnamyl-alcohol dehydrogenase (CAD) genes were overexpressed in the *L. dispar* herbivory group. Of fourteen peroxidase (POD) genes, six POD genes were up-regulated in the *L. dispar* herbivory group and in the mechanical wounding group while eight POD genes were suppressed compared with control group. A shikimate O-hydroxycinnamoyltransferase (HCT) gene was down-regulated in the *L. dispar* herbivory group and the mechanical wounding group, a beta-glucosidase (BGL) gene was up-regulated in the *L. dispar* herbivory group compared with the mechanical wounding group.

In the flavonoid biosynthesis pathway, five chalcone synthase (CHS) genes and two chalcone isomerase (CHI) genes were significantly regulated in the *L. dispar* herbivory group with 10.53 ~ 23.41-fold and 6.70 ~ 7.54-fold of those in mechanical wounding group, respectively. Compared with the control group, five flavanone 3-hydroxylase (F3H) genes were overexpressed in the *L. dispar* herbivory group and two of them were suppressed in the mechanical wounding group. Moreover, the expression levels of F3H in *L. dispar* herbivory group were 2.82 ~ 10.88-fold of those in mechanical wounding group. A flavonoid 3'-hydroxylase (F3'H) gene was induced in the *L. dispar* herbivory group with 6.07-fold of that in the mechanical wounding group. Except for one dihydroflavonol 4-reductase (DFR) gene, four DFR genes were up-regulated in the *L. dispar* herbivory group with 8.24 ~ 15.41-fold of those in the mechanical wounding group. Of three flavonol synthase (FLS) genes, one FLS gene was overexpressed in the *L. dispar* herbivory group and was suppressed in the mechanical wounding group. Two anthocyanidin synthase (ANS) genes were significantly induced in the *L. dispar* herbivory group compared with those in the mechanical wounding group. Five of six leucoanthocyanidin reductase (LAR) genes were overexpressed in the *L. dispar* herbivory group compared with those in the mechanical wounding group. An anthocyanidin reductase (ANR) gene was down-regulated in the *L. dispar* herbivory group and in the mechanical wounding group (Fig. 7).

Discussion Plants can reorganize their transcriptome, proteome and metabolome to deal with abiotic and biotic stress [11]. Plant resistance traits are extremely plastic against insect herbivores [12]. However, the protein and metabolite constituents of host plants are determined by the plant species, herbivore species, infested time, and detection techniques [13]. The *C. suppressalis* larvae herbivory results in 4729 genes and 151 metabolites differently regulated into rice plants. The defense-related phytohormones, transcript factors, and secondary metabolism mediated by shikimate and terpenoid were activated while the growth-related counterparts were suppressed after *C. suppressalis* herbivory [14]. Latexes protect the

Ficus carica from microbes and herbivores attack by toxic proteins and metabolites produced in immature fruit, young petioles and lignified trunks [15]. In present study, the specific DEGs and differential metabolites were confirmed according to transcriptomes and metabolomes of *P. simonii* × *P. nigra* in response to attack by *L. dispar* larvae. Gene expression analyses revealed that differently DEGs were involved into *L. dispar* herbivory.

Variable gene expressions resulted in reconfiguration of primary and/or secondary metabolism after insect infestation [16, 17]. After pre-infested by the *O. furnacalis*, DEGs in maize plants are associated with resistance to herbivores by affecting some metabolic pathways such as benzoxazinoids, phytohormones, volatile organic compounds (VOC) pathways [18]. Our results showed that a large amount of genes related to the biosynthesis of phenylpropanoid and flavonoid were activated in the herbivory group and some secondary substances obviously induced such as anethole, methylchavicol, chavicol, p-coumaryl alcohol and leucodelphinidin. Interestingly, twenty metabolites, such as lolicine A, cimicifugoside and vinblastine sulfate were specially associated with *L. dispar* infestation (Supplementary Table S3). In the phenylpropanoid biosynthesis pathway, one PAL, six 4CL, six CCR, six CCR, three CAD, six POD, and one BGL genes in poplar were up-regulated after *L. dispar* herbivory. For example, tobacco (*Nicotiana tabacum*) overexpressing *Arabidopsis* transcription factor, *AtMYB12*, enhanced expressions of key genes involved in the phenylpropanoid pathway, resulting in higher accumulation of flavonols [19]. PAL, CAD and 4CL are key enzyme and/or rate-limiting enzyme in lignin biosynthesis, which play an important role in plant responses to biotic and abiotic stresses [21, 22, 23]. As a scavenger of reactive oxygen species (ROS), POD is usually generated in early resistance responses of plants to a variety of pathogens through blocking oxidant-mediated programmed cell death [24, 25]. The 4CL3 in *Isatis indigotica* hairy roots elicited by methyl jasmonate (MeJA) was confirmed to esterize caffeic acid associated with lignan production using the transcriptional and metabolic analyses [26]. Oviposition by *Spodoptera exigua* increased *Solanum dulcamara* resistance to the subsequent feeding larvae using transcriptome and metabolome analyses. The genes involved in phenylpropanoid metabolism significantly overexpressed in previously oviposited *S. dulcamara* plants by lepidopteran herbivore *S. exigua* [12]. Moreover, profiles of volatile emission did vary in the indirect defenses of *Populus nigra* to leaf herbivory by lepidopteran species (*Lymantria dispar*, *Laothoe populi* and *Amata mogadorensis*) and beetles species (*Phratora vulgatissima* and *Chrysomela populi*) [27]. The transcriptome and metabolome analyses showed the ROS and calcium in early signaling, and salicylic acid (SA) and jasmonic acid (JA) mediated the defence regulation of *Arabidopsis thaliana* attacked by *Brevicoryne brassicae*. Secondary metabolites changes revealed defense against aphids, such as accumulation of 4-methoxyindol-3-ylmethyl glucosinolate [28]. Flavonoids synthesized by the phenylpropanoid pathway participate in multiple physiological and biochemical processes in plants. In the flavonoid biosynthesis pathway, our results showed five CHS, two CHI, five F3H, a F3'H, four DFR, a FLS, two ANS, five LAR, and an ANR genes in poplar were significantly induced in response to *L. dispar* herbivory. However, insect can change biochemical and physiology to combat with plant secondary metabolites. Our group reported effects of six characteristic poplar secondary metabolites (caffeic acid, salicin, rutin, quercetin, flavone, and catechol) and three mixtures (flavone + salicin, salicin + caffeic acid

+ catechol, flavone + catechol + caffeic acid) on performance and GST and P450 activities of *L. dispar* [29].

In conclusion, the transcriptome and metabolome analyses were conducted to generate a series of data set concerning *P. simonii* × *P. nigra* defense attacked by *L. dispar*. Specific genes and metabolic substances in poplar were induced by lepidopteran *L. dispar*. The phenylpropanoids and flavonoid biosynthesis pathway involved into the defense responses to *L. dispar*. The comprehensive networks of transcripts and metabolites provide novel insights into poplar defense mechanisms in this study.

Conclusions

In this study, the specific mechanism of poplar was investigated in response to *L. dispar* herbivory based on transcriptomic and metabolomic analyses. The defense responses of poplar are partially similar by *L. dispar* herbivory and mechanical wounding treatments. The 3666 specific DEGs were caused by *L. dispar* herbivory, and these specific DEGs are mainly enriched in phenylpropanoid biosynthesis pathway (ko00940) and flavonoid biosynthesis pathway (ko00941) by the analysis of KEGG metabolic pathway. The ubiquinone-6 and cimicifugoside showed significantly different expression in metabolome caused by *L. dispar* herbivory. In summary, the specific different genes and metabolites are involved in the poplar defense caused by *L. dispar* herbivory, and phenylpropanoid biosynthesis (ko00940) and flavonoid biosynthesis (ko00941) may be key defense pathways in poplar activated by *L. dispar* herbivory damage stress.

Methods

Plants and insects

The *P. simonii* × *P. nigra* was kindly provided by Prof. Jing Jiang from State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin, Heilongjiang, China. Briefly, *P. simonii* × *P. nigra* was clonally propagated in tissue culture, transferred to soil and grown in a mixture of peat and sand (2:1 v/v) in a greenhouse with day temperatures of 24°C and night temperature of 12°C conditions. No chemical sprays were used into the poplar seedlings during the all experiments.

Lymantria dispar eggs were initially obtained from the Research Institute of Forest Ecology, Environment, and Protection, CAF, and stored at 4°C before hatching. Larvae were reared on artificial diets at (25±1)°C under 14/10 h light/dark conditions, as described previously [30]. Healthy and hatching on the first day of 4th instar *L. dispar* larvae were randomly selected for subsequent experimental processing.

Herbivory and mechanical wounding treatments

Thirty healthy 4th instar *L. dispar* larvae were conducted into *P. simonii* × *P. nigra* seedlings without pests and diseases with the similar diameter (mean: 18.31mm±2.15mm) and height (mean: 110.27cm±3.79cm). After the poplar plants were placed into the greenhouse for 48 hours, the *L. dispar*

larvae pretreated with starvation for 6 h were uniformly transferred to the poplar plants, and the *L. dispar* larvae on the poplar were evenly re-distributed for four times per every day. To prevent larvae from escaping, the poplars were caged with a nylon net (Fig. 1). Corresponding to the treatment time and damage leaves of the *L. dispar* herbivory group, the poplar leaves was cut as mechanical wounding to simulate the *L. dispar* herbivory corresponding four times per day. After 24 h of treatment, all poplar leaves were used liquid nitrogen for quick freezing and stored in a -80°C refrigerator. The *L. dispar* herbivory group was abbreviated as GH, the mechanical wounding group was abbreviated as M, and the control group was abbreviated as CK. All treatments are performed in three biological replicates. One replicate was mixture of three technical replicate at equal ratios.

RNA extraction, library construction, and sequencing

The CTAB method was used to extract total RNA according to the manufacturer's instructions [31]. DNase I was used to clean out DNA contaminant in RNA samples. The mRNA molecules in total RNA were isolated using oligo (dT)-attached magnetic beads and were broken into small pieces using fragmentation reagent. First-strand cDNA was generated by random hexamer-primed reverse transcription, followed by synthesis of second-strand cDNA. The synthesized cDNA was subjected to end-repair and following 3'-adenylated. And the ends of these 3'-adenylated cDNA fragments ligated adaptors. PCR products were purified with the solid phase reverse immobilization (SPRI) beads and then were dissolved in EB solution. The double stranded PCR products were heating denatured and circularized by the splint oligo sequence. The single-strand circle DNA (ssCir DNA) was formatted as the final library. The library was confirmed by Agilent Technologies 2100 bioanalyzer, and was amplified with phi29 to form DNA nanoball (DNB) which have more than 300 copies of one molecular. The DNBs were loaded into the patterned nanoarray and single end reads of 50 bp were generated in the way of sequenced by synthesis. The library was used for sequencing through the sequencing BGISEQ-500 platform (BGI, Shenzhen, China).

De novo assembly and annotation

Prior to assembly, the reads containing adaptor, unknown base N content > 5%, and low-quality bases (>20% of the bases with a quality score ≥ 10) were removed by SOAPnuke (<https://github.com/BGI-flexlab/SOAPnuke>, the BGI, Shenzhen, China). After filtering, the remaining reads were called "clean reads" and stored in FASTQ (A format that stores biological sequences and corresponding quality assessments) format. *De novo* assembly of clean reads was performed using Trinity (v2.0.6, min_kmer_cov:3). Then, we used the TGI Clustering Tool (TGICL) to cluster transcripts to unigenes [32].

To acquire comprehensive information on gene functions, assembled unigenes were annotated using four databases, including the NCBI non-redundant (Nr) protein sequences, eukaryotic ortholog groups (KOG), SwissProt, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using BLASTx and BLASTn with an E-value $< 10^{-5}$. The gene ontology (GO) annotation was performed by Blast2GO with an E-value $< 10^{-5}$ based on the protein annotation results of the Nr database [33]. InterProScan5 was used

for interPro functional annotation [34]. The expression level of unigenes was calculated using FPKM (fragments per kilobase pairs per million mapped reads) values, which were indicated by running RSEM (RNA-Seq by Expectation Maximization) [35].

Metabolomics sample preparation

Chemical extraction was carried out on nine samples (three groups × three biological treatments) for metabolic analyses. Each sample aliquots (25 mg) were treated with 800 µL extraction liquid ($V_{\text{methanol}} : V_{\text{H}_2\text{O}} = 1:1$) and mixed well. The samples were ground using a tissue lyser (55 Hz, 5 min) into tubes where two magnetic beads were put into each tube, then were precipitated at -20°C for 2h. Samples were centrifuged at 25000 *g* for 20 min at 4°C, and supernatants were used for metabolomics analysis by ultraperformance liquid chromatography coupled to Q-TOF mass spectrometry (UPLC/Q-TOF MS).

UPLC/Q-TOF MS analysis

UPLC-MS analyses were conducted using an ACQUITY UPLC with a 2777C autosampler coupled to a Xevo G2-XS QTOF mass spectrometer (Waters, Manchester, U.K). An ACQUITY UPLC HSS T3 column (100mm×2.1mm, 1.8µm, Waters, Manchester, U.K) was used for reversed phase separation, and the column oven was maintained at 50°C. The mobile phase was solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) with 0.4 mL/min of the flow rate. Gradient elution conditions were set as follows: 0~2 min, 100% phase A; 2~11 min, 0% to 100% phase B; 11~13 min, 100% phase B; 13~15 min 0% to 100% phase A. The injection volume per sample was 10 µL.

The high-resolution tandem mass spectrometer was used to detect the metabolites eluted from the column. The Q-TOF was conducted in both positive and negative ion modes. The capillary and sampling cone voltages were set at 3.0 kV and 40.0 V in the positive (POS) ion mode, respectively. The capillary and sampling cone voltages were set at 2.0 kV and 40.0 V in the negative (NEG) ion mode, respectively. The mass spectrometry data were obtained in Centroid MSE mode. The TOF mass ranged from 50 to 1200 Da with 0.2 s of scan time. All precursors were fragmented using 20-40 eV for the MS/MS detection with 0.2 s of scan time. During the acquisition, the LE signal was acquired every 3 s to calibrate the mass accuracy. In addition, to evaluate the stability of the LC-MS during the whole acquisition process, a quality control (QC) sample was taken from every 10 samples.

Metabolomic data analysis

Data pretreatment of raw UPLC-MS data including peak picking, peak grouping, retention time (RT) correction, second peak grouping, and annotation of isotopes and adducts was performed using Progenesis QI software (Waters, Manchester, U.K) and the freely available R statistical language (metaX). LC-MS raw data files were initially converted into net CDF format, which is further processed by the XCMS and CAMERA toolbox. RT and the *m/z* data pairs were used as identifiers for each ion.

To obtain consistent variables, the resulting matrix was further reduced by removing peaks with more than 80% missing values (ion intensity = 0) and those with isotope ions from each group. To ensure the high quality of the data within the analysis process, an approach based on the periodic analysis of a standard biological QC sample and the true samples was used as a quality assurance strategy in metabolic profiling. Each retained peak was normalized to the QC sample by using robust Loess signal correction. The relative standard deviation (RSD) value of metabolites in the QC samples was set a threshold of 30% to assess the repeatability of the metabolic data sets. The logarithmic transformation and pareto scaling were applied to the datasets before statistical analysis.

Principal components analysis (PCA) was used to investigate and visualize the changes of metabolite in an unsupervised manner. Then, *t* test and fold change (FC) analysis (nonparametric univariate method [Mann-Whitney-Wilcoxon test]) were performed to measure the importance of metabolic characteristics from the preprocessed UPLC-MS datasets. The p-values generated by statistical test were adjusted to obtain q-values by using false discovery rate (FDR) correction. The screening requirement for differential metabolites was $FC \geq 1.2$ or ≤ 0.8333 .

Metabolite annotation and identification

The progenesis QI software was used for annotations and identification of metabolites. Briefly, the exact molecular masses and their fragmentation traits were annotated using public databases, Human Metabolome Database (HMDB; <http://www.hmdb.ca/>) and KEGG (www.genome.jp/kegg/) databases. Metabolites searched from databases were fragmented to obtain theoretical fragment spectrum. Then, the similarity scores were determined by comparing ion secondary spectrum from deconvolution of observed and theoretical spectra. The specific metabolites were screened according to the scores and difference between observed and theoretical mass. The isotopic distribution measurement was further used to identify the molecular formulae of matched metabolites.

Abbreviations

DEG: differentially expressed gene; LC-MS: liquid chromatography-mass spectrometry; PCA: principal component analysis; PAL: phenylalanine ammonia-lyase; 4CL: 4-coumarate-CoA ligase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl-alcohol dehydrogenase; POD: peroxidase; HCT: shikimate O-hydroxycinnamoyltransferase; BGL: beta-glucosidase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; DFR: dihydroflavonol 4-reductase; FLS: flavonol synthase; ANS: anthocyanidin synthase; LAR: leucoanthocyanidin reductase; ANR: anthocyanidin reductase; PCA: principal components analysis.

Declarations

Ethics approval and consent to participate

All plant materials used in our study were permitted and obtained from the State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University. Experimental research on plant, including collection of plant materials, complied with institutional, national, or international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The relevant datasets in this study are included within this article and the supplementary files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was financially supported by grants from National Key R&D Program of China (2018YFC1200400) and the Fundamental Research Funds for the Central Universities (2572019CG04) and Heilongjiang Touyan Innovation Team Program (Tree Genetics and Breeding Innovation Team). The funding bodies were not involved in the design of the study, data collection, interpretation of data, or in writing the manuscript.

Authors' contributions

CWC and YTL conceived and designed the research. YTL, JJY, YPJ and HD conducted experiments. YTL and CWC analyzed data. YTL, LLS and CWC wrote the manuscript. YTL, LLS and CWC discussed the results. All authors have read and approved the manuscript.

Acknowledgements

We thank Prof. Jing Jiang for providing *P. simonii* × *P. nigra* seedlings.

References

1. War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, et al. Mechanisms of plant defense against insect herbivores. *Plant Signal Behav.* 2012;7:1306–20.
2. Schoonhoven L, Loon JJ, Dicke M. *Insect-plant biology.* Oxford University Press. 2005:239–272.
3. Ryan CA. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Ann Rev.* 1990;28:425–49.
4. Hahlbrock K, Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. *Annu Rev Plant Biol.* 1989;40:347–69.

5. Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. *Plant Cell*. 1995;7:1085–97.
6. Tsai CJ, Harding SA, Tschaplinski TJ, Lindroth RL, Yuan Y. Genome-wide analysis of the structural genes regulating defense phenylpropanoid metabolism in *Populus*. *New Phytol*. 2006;172:47–62.
7. Li GL. Preliminary report on introduction of *Populus simonii* × *P. nigra*. *Technol Bull*. 1981;1:8.
8. Walley JW, Coughlan S, Hudson ME, Covington MF, Kaspi R, Banu G, et al. Mechanical stress induces biotic and abiotic stress responses via a novel cis-element. *PLoS Genet*. 2017;3:172.
9. Cao CW, Liu GF, Wang ZY, Yan SC, Ma L, Yang CP. Response of the gypsy moth, *Lymantria dispar* to transgenic poplar, *Populus simonii* × *P. nigra*, expressing fusion protein gene of the spider insecticidal peptide and Bt-toxin C-peptide. *J Insect Sci*. 2010;10:200.
10. Mochida K, Shinozaki K. Advances in omics and bioinformatics tools for systems analyses of plant functions. *Plant Cell Physiol*. 2011;52:2017–38.
11. Gulati J, Kim SG, Baldwin IT, Gaquerel E. Deciphering herbivory-induced gene-to-metabolite dynamics in *Nicotiana attenuata* tissues using a multifactorial approach. *Plant Physiol*. 2013;162:1042–59.
12. Geuss D, Lortzing T, Schwachtje J, Kopka J, Steppuhn A. Oviposition by *Spodoptera exigua* on *Solanum dulcamara* alters the plant's response to herbivory and impairs larval performance. *Int J Mol Sci*. 2018;19:4008.
13. Appel HM, Fescemyer H, Ehrling J, Weston D, Rehrig E, Joshi T, et al. Transcriptional responses of *Arabidopsis thaliana* to chewing and sucking insect herbivores. *Front Plant Sci*. 2014;5:565.
14. Liu QS, Wang XY, Tzin V, Romeis J, Peng YF, Li YH. Combined transcriptome and metabolome analyses to understand the dynamic responses of rice plants to attack by the rice stem borer *Chilo suppressalis* (Lepidoptera: Crambidae). *BMC Plant Biol*. 2016;16:259.
15. Kitajima S, Aoki W, Shibata D, Nakajima D, Sakurai N, Yazaki K, et al. Comparative multi-omics analysis reveals diverse latex-based defense strategies against pests among latex-producing organs of the fig tree (*Ficus carica*). *Planta*. 2018;247:1423–38.
16. Wu J, Baldwin IT. New insights into plant responses to the attack from insect herbivores. *Annu Rev Genet*. 2010;44:1–24.
17. Zhou S, Lou YR, Tzin V, Jander G. Alteration of plant primary metabolism in response to insect herbivory. *Plant Physiol*. 2015;169:1488–98.
18. Guo JF, Qi JF, He KL, Wu JQ, Bai SX, Zhang TT, et al. The Asian corn borer *Ostrinia furnacalis* feeding increases the direct and indirect defence of mid-whorl stage commercial maize in the field. *Plant Biotechnol J*. 2019;17:88–102.
19. Misra P, Pandey A, Tiwari M, Chandrashekar K, Sidhu OP, Asif MH, et al. Modulation of transcriptome and metabolome of tobacco by *Arabidopsis* transcription factor, *AtMYB12*, leads to insect resistance. *Plant Physiol*. 2010;152:2258–68.
20. Masaya K, Yoshitaka H, Hiroshi H, Yoshinori I, Masamichi Y. Wound-induced ethylene synthesis and expression and formation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase,

- phenylalanine ammonia-lyase, and peroxidase in wounded mesocarp tissue of *Cucurbita maxima*. *Plant Cell Physiol.* 2000;4:440–7.
21. Moura JC, Bonine CA, Oliveira FV, Dornelas MC, Mazzafera P. Abiotic and biotic stresses and changes in the lignin content and composition in plants. *Ann Rev Phytopathol.* 2010;360–376.
 22. Barakat A, Bagniewska-Zadworna A, Frost CJ, Carlson JE. Phylogeny and expression profiling of CAD and CAD-like genes in hybrid Populus (*P. deltoides* × *P. nigra*): evidence from herbivore damage for subfunctionalization and functional divergence. *BMC Plant Biol.* 2010;10:100.
 23. Uhlmann A, Ebel J. Molecular cloning and expression of 4-coumarate: coenzyme A ligase, an enzyme involved in the resistance response of soybean (*Glycine max* L.) against pathogen attack. *Plant Physiol.* 1993;102:1147–56.
 24. Marrs KA. The functions and regulation of glutathione S-transferases in plants. *Annu Rev Plant Physiol Plant Mol Biol.* 1996;47:127–58.
 25. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot.* 2003;91:179–94.
 26. Zhang L, Chen J, Zhou X, Chen X, Li Q, Tan H, et al. Dynamic metabolic and transcriptomic profiling of methyl jasmonate-treated hairy roots reveals synthetic characters and regulators of lignan biosynthesis in isatis indigotica fort. *Plant Biotechnol J.* 2016;14:2217–27.
 27. Fabisch T, Gershenzon J, Unsicker SB. Specificity of herbivore defense responses in a woody plant, black poplar (*Populus nigra*). *J Chem Ecol.* 2019;45:162–77.
 28. Kuśnierczyk A, Winge P, Jorstad TS, Troczynska J, Rossiter JT, Bones AM. Towards global understanding of plant defence against aphids—timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. *Plant, Cell Environ.* 2008;31:1097–1115.
 29. Wang ZY, Nur FA, Ma JY, Wang JG, Cao CW. Effects of poplar secondary metabolites on performance and detoxification enzyme activity of *Lymantria dispar*. *Comp Biochem Phys C.* 2019;225:108587.
 30. Cao CW, Sun LL, Wen RR, Shang QL, Ma L, Wang ZY. Characterization of the transcriptome of the Asian gypsy moth *Lymantria dispar* identifies numerous transcripts associated with insecticide resistance. *Pestic Biochem Physiol.* 2005;119:54–61.
 31. Zeng FS, Nan N, Zhan YG. Extraction of total RNA from mature leaves rich in polysaccharides and secondary metabolites of *Betula platyphylla* Suk. *Plant Physiol Comm.* 2007;43:913.
 32. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol.* 2011;29:644–52.
 33. Draghici S, Khatrri P, Tarca A, Amin K, Done A, Voichita, C. A systems biology approach for pathway level analysis. *Genome Res.* 2007;17:1537–45.
 34. Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, et al. InterProScan: Protein Domains Identifier. *Nucleic Acids Res.* 2005;33:116–20.
 35. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 2012;9:357–9.

Figures

Figure 1

Schematic diagram of poplar wounding treatment. All leaves were cut from three different treatments after 24 h.

Figure 2

The numbers of differentially expressed genes (DEGs) between different comparisons. GH-vs-CK, M-vs-CK and GH-vs-M represent *L. dispar* herbivory vs control, mechanical wounding vs control, *L. dispar* herbivory vs mechanical wounding, respectively.

Figure 3

The venn diagram of DEGs between three different comparisons. The red and blue arrows indicate up- and down-regulated DEGs, respectively.

Figure 4

KEGG pathway enrichment of DEGs between *L. dispar* herbivory group (3666 genes) and mechanical wounding group (1171 genes).

Figure 5

The venn diagrams of different ion number between different comparisons in pos and neg mode, respectively.

Figure 6

Metabolome variation in poplar leaves was determined among *L. dispar* herbivory group, mechanical wounding group and control group by the PCA method. (A) Metabolome variation among samples and top 10 metabolites for the PC2 under the control and *L. dispar* herbivory conditions in neg mode; (B) Metabolome variation among samples and top 10 metabolites for the PC2 under the control and *L. dispar* herbivory conditions in pos mode; (C) Metabolome variation among samples and top 10 metabolites for

- [TableS3.docx](#)
- [TableS2.docx](#)
- [TableS1.docx](#)
- [FigureS1S3.docx](#)