De novo assembly and comparative transcriptome analysis reveal genes potentially involved in changes of terpenoids in wheat germplasm resource, that defend against the wheat aphid Sitobion avenae (Fabricius)

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Abstract

Background

Terpenoid volatiles play an important role in direct and indirect plant defense responses against herbivores, including in gramineous crops such as transgenic rice and corn. The conventional varieties of wheat, an important gramineous cereal crop, lack aphid-resistant genes. It is therefore, necessary to seek aphid-resistant genes by screening for potential terpenoid synthase genes in wheat germplasm resources.

Results

The result showed that aphid-damaged Octoploid Tirtitrigia emitted a higher amount of S-linalool, ent-kaurene, (+)-delta-cadinene, (3S,6E)- nerolidol compared to intact plant. In addition, (E)-β-caryophyllene, β-Myrcene, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) were new volatile terpenoids emitted by the damaged plant. Further olfactory responses tests showed that S-linalool significantly repelled Sitobion avenae (Fabricius). Using the Illumina sequencing platform, approximately 203.09 million high quality paired-end reads were obtained. After de novo assembly and quantitative assessment, a total of 182,348(74.8%) unigenes were annotated by alignment with public protein databases. Of these unigenes, 2,389 differentially expressed genes were identified between intact and damaged ears of Octoploid Trititrigia. The expression profile of 10 randomly selected TPSs was confirmed with RT-qPCR. Candidate genes involved in terpenes biosynthesis were identified showing significant transcript changes between intact and damaged plant ears of Octoploid Trititrigia. Also transcript abundances of terpenes biosynthetic pathway-related genes were positively correlated with the production of volatile terpenoids in ears. The unigenes of S-linalool synthase gene was mapped to the cloned cDNA WT008_M07 (AK333728) and WT013_P07 (AK335856) of the Chinese spring wheat cultivar. The predicted protein complete ORF sequence (TaLIS1/2) when compared with the S-linalool synthase gene of other species, contained an aspartate-rich region DDxxD motif. Its function was characterized as coordinating the divalent metal ions involved in substrate binding. Furthermore, phylogenetic tree results showed that the TaLIS1 and TuNES1 are highly homologous.

Conclusions

This assembled transcriptome of S. avenae-damaged Octoploid Trititrigia and intact ears can provide more molecular resources for future functional characterization analysis of genomics in volatile terpenoids involved in direct or indirect defenses. Our study describes the metabolic regulation mechanism of volatile terpenoids in gramineous crops, which provides support for both breeding and genetic modification of wheat varieties resistant to wheat aphids.

Background

Plants interact with the environment by producing a variety of chemical compounds. Plants can produce volatile compounds to protect themselves when attacked by herbivorous insects [1;2]. Herbivore-induced plant volatiles (HIPVs) help plants protect themselves from herbivores that might cause mortality or reduced reproductive fitness. HIPVs can deter herbivores in the field [3–5] or laboratory [6–8], and attract natural enemies of an herbivore [9–12]. Yet, herbivores can also be attracted to plant volatile compounds induced by herbivory or pathogen infection [13, 14].

Volatile terpenoids are the major component of HIPVs, and mainly classified as monoterpenes, sesquiterpenes, and homoterpenes. A well-studied example of the role of volatiles in plant defense is the tri-trophic interaction among lima bean (*Phaseolus lunatus*Linn.), spider mites (*Tetranychus urticae* Koch) and predatory mites (*Phytoseiulus persimilis*Athias-Henriot). When lima bean plants are damaged by spider mites, *P. lunatus* leaves release a complex blend of volatiles, especially volatile terpenoids which can attract the natural enemy, *P. persimilis [*15]. Studies have shown that in maize (*Zea mays,* Linn.), damage by lepidopteran larvae such as *Spodoptera littoralis* Boisduval induces a complex volatile blend that attracts its natural enemies, the parasitic wasps [16–19]. Terpene synthases (TPSs) in plants were mainly through the mevalonate (MVA) pathway in the cytoplasmic/endoplasmic reticulum or the 2-c-methyl-d-erythritol 4-phosphate (MEP) pathway in the plastids to synthesize terpenes. And the geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranyl diphosphate (GGPP) were converted into mono-, sesqui- and diterpene skeletons respectively [21]. Based on phylogeny and functional studies, TPSs are commonly divided into eight subfamilies: TPS-a (sesquiterpenes), TPS-b (cyclic monoterpenes and hemiterpenes), TPS-c (copalyl diphosphate synthases), TPS-d (gymnosperm-specific), TPS-e (ent-kaurene synthases), TPS-f (other diterpene synthases), and TPS-h (lycopod-specific) [22]. There is a medium-sized gene family for most plant genomes with encoding terpene synthetases. It is important for studying the physiological and ecological functions of specific terpenoids by using plants with overexpressing the TPS gene [15, 23–26]. For instance, expressing *FaNES1* of the strawberry (*Fragaia* × *ananassa*Duch.) linalool/(E)-nerolidol synthase, into *Arabidopsis thaliana* (Linn.) mitochondria, increased the production of (E)-nerolidol and DMNT and attracted the carnivorous predatory mites [27]. The overexpression in *A. thaliana* of wheat FPP synthase gene (FPS) could repellent the peach potato aphid (*Myzus persicae* Sulzer) [26]. Overexpression of *P. lunatus* TPS genes *Pltps3* and *Pltps4* in rice increased the attraction of transgenic rice plant to the natural enemies of the rice stemborer (*Chilo suppressalis* Walker) [15]. Transgenic *Nicotiana tabacum* Linn. plants overexpressing *GhTPS12* gene, which produced relatively large amounts of (3S)-linalool, showed direct defense against herbivores [28]. Studies have shown that S-linalool is the most abundant volatile emitted by rice plants damaged by caterpillars of the fall armyworm (*Spodoptera frugiperda*Smith & Abbot), and could significantly attract parasitic wasps [29]. S-linalool is a key component in the direct and indirect induced volatiles of rice. In the defense response against brown planthopper (*Nilaparvata lugens (*Stal)), rice adopts the “push- pull” strategy using S-linalool as the weapon to drive away the pest *N. lugens* and attracting the natural enemy, the parasitoid *Anagrus nilaparvatae* Pang & Wang [11]. Many TPSs are multiproduct enzymes, which produce volatile terpenoids that can play a direct or indirect defense against herbivory.

The English green aphid, *Sitobion avenae* (Fabricius), is considered one of the most important pests of wheat in Asia and Europe [30–32]. It can cause heavy economic damage to wheat, both as a phloem feeder and as a vector of plant viruses [33–35]. It was reported that the wheat (*T. aestivum,* AABBDD) gene *Tafps1* and *Tafps2* played important roles in induced responses to aphid infestation and in sesquiterpene synthesis [26]. The alarm pheromone for many pest aphids, the sesquiterpene (E)-β-farnesene, was successfully developed by genetic engineering of the hexaploid wheat cv. Cadenza. The released pheromone showed intrinsic activity against aphid pests and attracted the natural enemy, the parasitic wasp *Aphidius ervi* Haliday in laboratory experiments. Although these studies show considerable potential for aphid control, there was no change in aphid numbers in field trials. This was attributed to low insect numbers and erratic climatic conditions [36]. However, volatiles terpenoids play an important role in direct and indirect plant defense responses against herbivores, especially in transgenic gramineous crops such as rice and corn [15, 24, 38]. Moreover, studies have shown that the expression of terpene synthase genes in plants increased dramatically after pest infestation [15, 39, 40]. Conventional wheat varieties emit few volatile terpenoids which showed resistance to aphids. Our previous study showed that the wheat germplasm resource Octoploid Trititrigia was a promising candidate for breeding aphid-resistant wheat varieties. The quality and quantity of volatile terpenoids changed remarkably in aphid-damaged wheat plant compared with intact wheat plant. In order to regulate the synthase genes of volatile terpenoids which showed resistance to aphids, we conducted a de novo transcriptome analysis to compare RNA-seq profiles between intact and damaged wheat plant ears of Octoploid Trititrigia by using Illumina sequencing technology. The purposes of our study were to investigate differentially and specifically expressed genes which related to the biosynthesis of volatile terpenoids, revealing a greatly enriched pathway for the changes of volatile terpenoids between intact ears and damaged ears of Octoploid Trititrigia. In addition, rely on the analysis of the transcript abundance of differentially expressed genes (DEGs), those candidate genes which involved in TPSs were also mined. The collected volatile terpenoids were analyzed with transcriptome data. The results could provide resources and candidate genes for breeding uniform resistant varieties to aphids

Results

Emission of terpenoids from aphid damaged Octoploid Trititrigia

GC-MS analysis of volatile compounds showed that aphid-damaged Octoploid Tirtitrigia emitted a higher amount of linalool, ent-kaurene, (+)-delta-cadinene and (3S,6E)-nerolidol compared to uninfested plants. In addition, the infested wheat plant emitted new HIPVs including (E)-β-caryophyllene, β-Myrcene and DMNT (Fig. 1).

Illumina sequencing and de novo assembly

We used the samples of the Octoploid Trititrigia ear, and produced 42.6 Gb of data with Illumina NovaSeq6000 high-throughput sequencing techniques. We obtained 243,718 high-quality reads for de novo assembly by trimming the adapter and primer sequences, fuzzy nucleotides, and low quality sequences. The total length of the transcribed sequence was 20,308,8986 bp, the average length was 833.29 bp, and the N50 size was 1041 bp with 47.09% GC content (Table 1). These assembled transcript sequence length was ranged from 300 to 3000 bp, and the dominate length of sequences was 300 to 400 bp (Fig. 2). In addition, the sequence length ≥3000bp transcripts numbers were 3863.

Functional annotation and classification

To obtain comprehensive gene function information, annotating unigenes into the NR (NCBI non-redundant protein sequence) public database, Swissport, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), KOG, NT (NCBI nucleotide sequences), Pfam. A total of 182,348(74.8%) unigenes had significant BLAST hits in at least one of the seven databases. Among them, 85,145 (34.94%), 42,318 17.36%), 53,526 (21.96%), 125,347 (51.47%), 138,277 (56.74%), 68,267 (28.01%) 118,398 (48.58%) unigenes were blasted to GO, KEGG, KOG, NR, NT, Swissport and Pfam respectively

Gene ontology (GO) classification system was used to classify the functions of the annotated genes. GO divided into three categories, namely biological process, molecular function and cellular component. A Total of 85,145 unigenes were assigned to 52 GO terms at the second level (Fig. 3).

KOG analysis showed that a total of 53,526 non-redundant unigenes with high homology were grouped into 25 functional classes. “General function prediction only” (13,453, 25.13%), “Signal transduction mechanisms” (6,669, 12.46%), “Posttranslational modification, protein turnover, chaperones” (5,573, 10.41%) were the three largest categories (Fig. 4, Additional file 1).

KEGG is a database that systematically analyzes the inner-cell metabolic pathways and functions of gene products, which are important for studying the complex biological behavior of genes. Totally 42,318 unigenes were mapped onto 133 pathway, and the most enriched sequences were “Ribosome” (1,749, 4.13%), followed by “carbon metabolism” (1,738, 4.11%), “Biosynthesis of amino acids” (1,490, 3.52%), and “Plant-pathogen interaction” (1,334, 3.15 %). “Carbapenem biosynthesis”, “Indole alkaloid biosynthesis”, “Isoflavonoid biosynthesis” each contained only two members (Additional file 2).

Identification and functional enrichment analysis of DEG

Based on the FPKM method, the transcript abundance of each gene from intact Octoploid Trititrigia ears and aphid-damaged ears was analyzed. A total of 233,023 DEGs were identified between intact and damaged Octoploid Trititrigia ear libraries (Additional file 3). Out of these DEGs, 2,389 genes exhibited a significant difference in their expression levels with the threshold of FDR ≤ 0.05, including 2,306 up-regulated and 83 down-regulated ones (Fig. 5, Additional file 4). These identified DEGs were annotated in

GO categories. According to Bonferroni-corrected P value≤0.05, significantly enriched GO terms in DEGs were enriched in three main categories of biological process, molecular function and cellular component (Fig. 6, Additional file 5). The terms of “catalytic_activity” (GO:0003824), “purine_ribonucleoside_binding” (GO:0032550), “adenyl_ribonucleotide_binding” (GO:0032559), “hydrolase_activity” (GO:0016787), “ribonucleoside_binding” (GO:0032549), “metal_ion_binding” (GO:0046872), “purine_nucleotide_binding” (GO:0017076), “carbohydrate_derivative_binding” (GO:0097367) were the dominant groups in the molecular functions; “ribosome” (GO:0005840), “cytosol” (GO:0005829), “cell_part” (GO:0044464), “intracellular” (GO:0005622), “membrane_part” (GO:0044425), “cytoplasm” (GO:0005737), “intracellular_part” (GO:0044424), “intracellular_organelle_part” (GO:0044446), “organelle_part” (GO:0044422) were the representative groups in the cellular components. Among the biological processes, a great number of DEGs were categorized under “single-organism_process” (GO:0044699), “nitrogen_compound_metabolic_process” (GO:0006807), “single-organism_metabolic_process” (GO:0044710), “translation” (GO:0006412), “localization” (GO:0051179), “transport” (GO:0006810), “establishment_of_localization” (GO:0051234) and “small_molecule_metabolic_process” (GO:0044281).

Furthermore, 2,389 of the significantly DEGs were blasted to the KEGG database to analyze their biological pathways. Among 269 of the enriched KEGG pathways (Additional file 6), the most significant ones (p-value ≤ 0.05) consisted of “Ribosome”, “Monoterpenoid biosynthesis”, “Cardiac muscle contraction”, “Alzheimer’s disease”, “Huntington’s disease”, and “Protein processing in endoplasmic reticulum” (Table 2; Fig. 7). This was followed by “Parkinson’s disease” (KO05012), “Phenylpropanoid biosynthesis” (KO00940), “Proteasome” (KO03050), “Nitrogen metabolism” (KO00910), “Non-alcoholic fatty liver disease” (NAFLD) (KO04932), “Carbon metabolism” (KO01200), “Citrate cycle” (TCA cycle) (KO00020) and “Oxidative phosphorylation” (KO00190).

Candidate genes related to terpene biosynthesis

Terpene synthases (TPSs) are the key enzymes in the terpene biosynthesis pathway. In the present study, changed transcript abundances of TPSs gene were detected between intact and aphid damaged ears of Octoploid Trititrigia (Additional file 7). The levels of transcripts encoding the TPSs biosynthesis, including Terpenoid backbone biosynthesis, Monoterpenoid biosynthesis, Diterpenoid biosynthesis, Ubiquinone and other terpenoid-quinone biosynthesis and cytochrome P450 of aphid damaged Octoploid Trititrigia ears were significantly higher than those of intact Octoploid Trititrigia ears. These were further authenticated by RT-qPCR. The levels of transcripts encoding terpene biosynthesis, including annotation in databases of Myrcene synthases, cytochrome P450 oxidase, (3S,6E)-nerolidol synthases, 3S-linalool synthases and (+)-delta-cadinene synthases genes, were significantly higher in aphid damaged Octoploid Trititrigia than those of intact Octoploid Trititrigia ears (Fig. 8)

Sequence analysis of Octoploid Trititrigia predicted 3S-linalool synthase gene

GC-MS combined with transcriptome analysis results showed that S-linalool was the most abundant volatile emitted from aphid damaged Octoploid Trititrigia ears. The assembly sequence of S-linalool synthase gene was analyzed, in which most of the transcript annotated with S-linalool could be mapped to the cDNA sequences WT008_M07 (AK333728) and WT013_P07 (AK335856) of the Chinese Spring wheat cultivar in NT database. The complete ORF of *TaLIS*1 (WT008_M07) was 1278bp, within a predicted Mw (molecular weight) of 49.01 kDa, ecoding 425 amino acids, and had a pI (isoelectric point) of 6.23. In addition, the complete ORF of *TaLIS*2 (WT013_P07) was 1278 bp, a predicted Mw of 63.14 kDa and a pI of 5.88 which encoded by 454 amino acids. The amino acid sequence alignment of *TaLIS1* and *TaLIS2, Aegilops tauschii LIS* (XP_020179676), *Oryza sativa LIS* (XP_015623808), *Zea mays LIS (*AQK60700), *Triticum urartu LIS* (EMS50987), *Arabidopsis thaliana LIS* (NP_176361) revealed typical conserved motifs and domains of specific terpene synthase (Fig. 9). *TaLIS* contained an aspartate-rich region DDxxD motif, which was described as a function involved in substrate binding to coordinate divalent metal ions [24].

TaLIS was clustered with angiosperm monoterpene synthases (TPS-g family) based on the phylogenetic relationship analysis (Fig.10). There was no difference in the phylogenetic relationship of the *TaLIS* gene family from that of the current plant. Furthermore, the S-linalool synthase of the close relatives almost clustered into a group, indicating that they have a closely evolutionary relationship in related plant species other than distant plant species.

Olfactory response of *S. avenae* to S-linalool

In order to determine the response of *S. avenae* to S-linalool, its olfactory responses todifferent doses of S-linalool were investigated. The results showed that when S-linalool concentration was 100 $\mu L \cdot mL^{-1}$, it had a significant repellent effect on aphids ($W = -3.385$, $P < 0.05$) (Fig. 11).

Discussion

The results of this study showed that the *S. avenae*can induce biosynthesis of volatile terpenoids such as S-linalool, DMNT, (E)-β-caryophyllene with significant ramifications for plant-insect interactions. Specifically, higher levels of S-linalool were detected in Octoploid Trititrigia ears infested by*S. avenae* compared with uninfested ears. Previous studies have shown that S-linalool was the most abundant volatile emitted from pest-damaged rice and cotton plants, and showed direct or indirect defense against herbivores [28, 29]. Also, *A. thaliana* plants genetically engineered to release linalool, repelled aphids [7]. Additionally, field studies conducted with *OsLIS*-silenced rice plants showed that inducible (3S)-linalool attracted predators, parasitoids as well as chewing herbivores, but repelled the rice brown planthopper *Nilaparvata lugens* (Stal)) [11]. The olfactory response of *S. avenae* indicated that the high concentration of S-linalool (100 $\mu L \cdot mL^{-1}$) had remarkable repellent effect on *S. avenae.* Further investigation would be needed to test the ecological functions of S-linalool on the aphid natural enemies such as *Aphidius gifuensis* Ashmaed and *Aphidius avenae* Haliday. Apart from S-linalool, the amount of ent-kaurene, (+)-

delta-cadinene were also significantly higher in *S. avenae*-damaged plant ears than intact ears. Likewise, the infested ears emitted new HIPVs such as β-Myrcene, (E)-β-caryophyllene and DMNT. Previous studies had shown that DMNT, (E)-β-caryophyllene, (+)-delta-cadinene could act as a direct or indirect defense substances against pests on plants [11, 23, 37, 38]. The functional significances of the other volatile terpenoids to aphids remain to be determined.

Some of the structural genes of TPSs have been identified, and even successfully used to increase direct plant defense [8, 28] or indirect plant defense by attracting natural enemies of the herbivores [9–11, 41]. Many structural genes involved in TPSs have been discovered in field crops in the past few years [15, 36, 42, 43], which has greatly promoted understanding of the modulation of terpene compounds biosynthesis. Meanwhile, many studies have reported that the expression levels of TPSs and cytochrome P450 enzyme were significantly correlated with pest defense in plants [15, 28, 38]. The emission of volatile terpenoids were higher in *S. avenae*-treated Octoploid Trititrigia ears than untreated ears. However, little has been known about the molecular mechanisms controlling the TPSs biosynthesis in wheat. Thus, a comprehensive identification of DEGs and modulating pathways related to the TPSs was profiled using de novo transcriptome data via comparison between intact and aphid damaged Octoploid Trititrigia ears.

TPSs and their volatile compounds, volatile terpenoids, play an important role in direct and indirect plant defense responses against herbivores [10, 44, 45]. Volatile terpenoids are generally released until plants have been damaged by herbivorous insects for some time [4, 35]. In this work, changed transcript abundances of the TPSs gene were detected in damaged Octoploid Trititrigia ears compared to intact Octoploid Trititrigiaplant ears. Based on RT-qPCR results, the expression profiles of the 10 genes were identical by Illumina sequencing or RT-qPCR analysis, but the accurate folding change of the two methods was biased. This suggested a relative rationality and accuracy of the transcriptome analysis in the present study.

In this study, TPSs and cytochrome P450 enzyme related genes displayed significant expression changes between the intact and aphid-damaged Octoploid Trititrigia tissues, which is in accord with previous findings about changes of volatile terpenoids in other plants after pest infestation [27]. Such information would help provide a deeper understanding of how changes in gene expression are related to the volatile terpenoids changes in the Octoploid Trititrigia ears.

The GC-MS analysis of volatile terpenes was combined with transcriptome data. The results showed that the quality and quantity of terpenoids volatile compounds in *S. avenae*-damaged ear changed compared to intact ear. Linalool was the most abundant volatile in *S. avenae* damaged compared to intact Octoploid Trititrigia (Fig.1). The transcripts regulating S-linalool synthase genes were also the most abundant. Most of the unigene of S-linalool synthase can be mapped to the cloned cDNA WT008_M07 (AK333728) and WT013_P07 (AK335856) of Chinese spring wheat cultivar, and the predicted protein sequence can be compared with the S-linalool synthase gene of other species. Phylogenetic tree results showed that the *TaLIS1* and *TuNES1*werehighly homologous. TPSs are multifunctional enzyme [23]. S-linalool synthase genes at the same time also can control (3S,6E)-nerolidol (DMNT precursor substances)

[15]. Therefore, the two *T. aestivum* synthase genes may also control S-linalool and (3S,6E)-nerolidol synthesis. GC-MS results showed that the components of volatile terpenes (3S,6E)-nerolidol and its ramification DMNT were detected. The S-linalool synthase gene function need further verification.This assembled Octoploid Trititrigia ear transcriptome can be used as a database of wheat germplasm resources to search for high quality aphid-resistance genes and provide more molecular resources for future functional characterization analysis of aphid resistance.

Conclusion

In conclusion, GC-MS analysis showed that the quality and quantity of volatile terpenoids from Octoploid Trititrigia varied dramatically after aphid damage. The transcriptome dataset of Octoploid Trititrigia showed that 243,718 unigenes were totally identified and annotated 182,348 unigenes (74.8%). Meanwhile, 2,389 DEGs were screened between the intact and the damaged ears. This assembled transcriptome of Octoploid Trititrigia could improve the functional characterization genomic analysis by providing more molecular resources in response to *S. avenae* infestation. Furthermore, it has been identified that the transcription levels of the TPSs family genes in damaged plant ears were higher than those in intact plant ears, which was consistent with the volatile constituents of terpenoids. Our results could get insight into the mechanism of *S. avenae* infestation under different metabolic processes. This will enable the genetic manipulation of Octoploid Trititrigia to strengthen aphid resistance.

Methods

Insects and plants

The colony of wheat aphid, *S. avenae,* were reared on seedlings of wheat lumai 21 in an environmental chamber (21°C, L16:D8 photoperiod, and 70% relative humidity), with new wheat seedlings provided once a week. Octoploid Tirtitrigia, the new intermediate type of wheat, which was breeding by introduced the genome of Thinopyrum intermedium (*Elytrigia intermedia (*Host) Nevski) into common wheat. Octoploid Tirtitrigia was provided by Dr. Xinfeng Li from National Wheat Improvement Center of China. We declare that the collection of plant materials comply with institutional, national, or international guidelines.

Treatments

Wheat Octoploid Tirtitrigia was sown in 10th October and covered by the insect-proof screen with 120 mesh. In the flowering stage, 100 adult wingless aphids of *S. avanae* were introduced in the ear of a plant to serve as treatment. Plants without aphids served as control. Both intact and damaged wheat ears were sampled after 72 h.

Volatile Collection and GC-MS Analysis

Sampling ears were collected in the field after 72 h infestation. the air entrainment (dynamic headspace collection) was carried out following standard procedures using the flowering ears of Octoploid Tirtitrigia. For each headspace collection, a ear was enclosed in a gas sampling bag (Polytetrafluoroethene, 2 L, E-Switch), open at the bottom, and with two collection ports at both sides (one for inlet of air and other for outlet), The bottom was closed without pressure around the plant stem, using seal the strip. Air, purified by passage through an activated charcoal filter, was pumped into the vessel through the inlet port at 600 mL·min^{-1} (flow rate was measured by a flow meter). Air was drawn out at 400 mL·min^{-1} through 50 mg Porapak Q (Alltech Associates, Carnforth, Lancashire, UK) in a 5 mm diam Tenax glass tube. The differences in flow rates created a slight positive pressure to prevent unfiltered air enter the system. All connections were made with polytetrafluoroethylene (PTFE) tubing with brass ferrules and fittings and closed with PITE tape. Porapak Q filled tubes were conditioned before use by washing with n-hexane (1 mL) and heating inside an oven (150 ℃) under a stream of nitrogen for a minimum of 2 h. VOCs were collected on Porapak Q tubes inserted into the collection port on the top of the vessel and were subsequently eluted with 500 μL freshly n-hexane. Air entrainment samples were analyzed by GC–MS. For air entrainment samples, aliquots of samples (1μL) were analyzed. All analyses were carried out using a DB5-MS column (30 m × 0.25 mm ID × 0.25 μm film thickness; Agilent Technologies) with a split/splitless injector (splitless mode, 220 °C). Helium was used as the carrier gas at 1 mL·min–1; the oven temperature was programmed to rise from 50 to 60°C (5 min hold) with a rate of 5°C min^{-1} and then raised to 250 °C with a rate of 10°C min^{-1} (5 min hold). The transfer line temperature was 250 °C; ion source temperature was 250°C. Ionization was by electron impact (70 eV), and the scan range was between m/z 50 and 650. Volatiles were identified by comparison of their GC retention times and mass spectra with authentic reference.

RNA isolation, cDNA library preparation and sequencing

Total RNA was extracted from three biological replicates of the flowering of earusing the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions, and then treated with RNase-free DNase I (Invitrogen). The quality and concentration of the total RNA were determined using a NanoPhotometer® spectrophotometer (Implen, CA, USA) and the Qubit® RNA Assay Kit with a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA), respectively. The RNA integrity was confirmed using an Agilent 2100 Bio-Analyzer (Agilent Technologies, CA, USA). RNA samples with RIN ≥7 and 28S:18S ratio ≥1.5:1, total amount ≥3μg, were considered acceptable and used as input material to construct the sequencing library. Illumina paired-end library was prepared using the standard TruSeq RNA Sample Prep Kit (Illumina Inc.). Briefly, poly (A) mRNA was enriched from total RNA using Sera-mag Magnetic Oligo (dT) Beads (Thermo Fisher Scientific, USA) according to Illumina manufacturer's instructions. Double-stranded cDNA was generated using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, USA). After that, Pair-end RNA-seq libraries were constructed using the Illumina Paired End Sample Prep kit and were then sequenced on an Illumina HiSeqTM 2000 system. High-quality paired end reads with a length of 200bp were obtained by deleting low-quality reads with vague nucleotides and filtering adapters from the raw data.

De novo transcriptome assembly, functional annotation and classification

For transcriptome assembly, a strict Illumina pipeline was used for filtering the raw sequence reads. All reads with adapter sequences, unknown nucleotides comprising more than 10%, and low-quality reads (> 50% base with quality value Q≤5 in a read) were removed. De novo transcriptome assembly was accomplished from all these clean reads with the Trinity program. Only those sequences with perfect homology or not more than two nucleotide mismatches were used for conservative and accurate annotation.

To annotate the transcriptome, assembled sequences were further used as query sequences to blast with Nr (NCBI non-redundant protein sequences), SwissProt, KEGG (Kyoto Encyclopedia of Genes and Genomes database) and KOG (eukaryotic orthologous groups), Pfam (Protein family) and GO databases, respectively. The best hit of alignment was used to infer biological function of assembled transcripts. Additionally, GO (Gene Ontology) terms of assembled transcripts were extracted from the best hits against the Nr and Pfam using the Blast2GO. After acquiring the GO annotation for each assembled transcript, GO functional classification was achieved using WEGO software for all the transcripts. KEGG pathway annotations were retrieved from KEGG (http://www.genome.jp/kegg/) database.

Identification of differentially expressed genes

Using the de novo assembled transcriptome data as reference sequence, the clean reads of each sample were mapped to this reference sequence using Bowtie 2, allowing no more than two nucleotide mismatches. The gene expression levels were determined by the numbers of reads uniquely mapped to the specific gene and the total number of uniquely mapped reads in the sample and calculated using the RPKM method (reads per kb per million reads). Using the edgeR software, differentially expressed genes (DEG) were determined between the damaged and intact Octoploid Tirtitrigia ear libraries, respectively, and the DEGs were defined as significant based on a false discovery rate (FDR)≤0.05 and an absolute value of log2Ratio≥1 [46].

GO and KEGG enrichment analysis of DEGs

The differentially expressed genes (DEGs) were used for GO function and KEGG pathway enrichment analysis, and a Bonferroni-corrected p value≤0.05 was selected as a threshold level to determine significant enrichment of DEGs. GO enrichment was conducted using Blast2GO and WEGO. KEGG enrichment analysis was performed using KOBAS based on the comparative results between the identified genes and the background reference sequences. GO terms and pathways enriched in the set of DEGs were calculated by the hypergeometric test.

RT-qPCR validation of DEGs

Ten terpene synthases (TPSs) genes that were prominently and differentially expressed in our expression profile data were randomly chosen for validation by reverse transcription quantitative PCR (RT-qPCR). Ten differentially expressed candidate genes related to TPS were selected for validation through RT-qPCR, and gene-specific primers were designed by Primer Premier 5. All primer pairs for these qPCR were deposited in Additional (Additional file 9). The qPCR use Top Green qPCR SuperMix (TransScript) in 20 μL reactions according to the manufacturer's instructions. The qPCR amplification was performed in thermocycler conditions starting with 30s at 94 °C, 5s at 94 °C, 40 cycles of 30s at 60 °C, and 10 s at 95 °C, followed by 1 min at 60 °C.The *T. aestivum* gene *Taactin* was taken as an internal reference. To calculate the expression abundances of target genes, the method $2^{-\Delta\Delta Ct}$ for statistics was applied with three biological replicates.

Sequence analysis of Octoploid Trititrigia predicted S-linalool synthase gene

The theoretical isoelectric point (pI) and molecular weight (MW) of the Octoploid Trititrigia S-linalool synthase gene was analyzed by the ExPASy website (http://web.expasy.org/compute_pi/). The protein coding region of Octoploid Trititrigia S-linalool synthase gene was searched by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Multiple sequence alignment of TPS protein sequences was performed by CLUSTALW. To reconstruct phylogenetic relationship, the neighbour-joining (NJ) tree was conducted using MEGA7.0 and 1 000 replicates of bootstrap analyses.

Four-arm olfactometer assays

Four-arm olfactometer were used to test the behavioural responses of aphids towards the S-linalool chemical compounds (Shyuanye, Shanghai, 98%). The olfactometer was made up of Plexiglas and possessed a 10.5 cm walking area that was similar to the one described by Vet et al. (1983). The four-arm olfactometer was connected to a vacuum pump to extract air, and the flow rate of each arm was 150 mL ⋅min^{-1}. The olfactometer assays were conducted in a temperature of (21 ± 1) ℃ and a relative humidity of (70 ± 5) % in controlled room. Five amounts (0.01,0.1,1,10,100 μL⋅mL^{-1}) of S- linalool, were placed on a 1cm x 1cm piece of filter paper and offered to the tested aphids. Each aphid was placed at the center of the exposure chamber, which was observed during 15 min and record the time which was each insect in every olfactometer areas. Twenty replicates were performed with aphids. Every 2 observations, the olfactometers were rotated by 90 degrees to avoid directional bias. Between each treatment, the system was cleaned with pure ethanol and rinsed with distilled water. Test data was expressed as "Mean + Standard Deviation", and IBM SPSS 20.0 was used for corresponding analysis and test. The nonparametric test (Wilcoxon signed rank sum test, and the test quantity was expressed by W)

for the two correlated samples was performed for the aphid residence time in the treatment arm and 1/3 of the residence time in the control arm.

Abbreviations

HIPVs: herbivore-induced plant volatiles; TPSs: terpene synthases; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; RT: Reverse transcriptase; ORF: Open reading frame; LIS: linalool synthase; NES: nerolidol synthase; GC-MS: Gas Chromatography-Mass Spectrometer;

Declarations

Ethics approval and consent to participate

Not applicable. Neither human or animal subjects, human or animal materials nor human or animal data were used on this manuscript. The authors declared that experimental research works on the plants described in this paper comply with institutional, national and international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

Part of the data generated or analysed during this study are included in this published article and its supplementary information files. The datasets generated transcriptomic analysis data during the current study are not publicly available due research period but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contribution

LZ and YL conceived and designed the experiments, LZ, ZL and YZ performed the experiments and analyzed the data. LZ and YL wrote the paper. All authors read and approved the manuscript.

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References

1. Clavijo MA, Unsicker SB, Gershenzon J. The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends in plant science. 2012;17:303–310.
2. Gols R. Direct and indirect plant defences against insects in a multitrophic framework. Plant Cell and Environment. 2014;37:8.
3. D'Alessandro M, Turlings TCJ. Advances and challenges in the dentification of volatiles that mediate interactions among plants and rthropods. Analyst. 2006;131:24–32.
4. De Moraes CM. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature. 2001;410:577–80.
5. Kessler A, Baldwin I T. Defensive function of herbivore-induced plant volatile emissions in nature. Science. 2001;291:2141–2144.
6. Abassi SA, Birkett MA, Pettersson J, Pickett JA, Wadhams LJ, Woodcock CM. Response of the seven-spot ladybird to an aphid alarm pheromone and an alarm pheromone inhibitor is mediated by paired olfactory cells. Journal of Chemical Ecology. 2000;26:1765–1771.

7. Aharoni A. Terpenoid Metabolism in Wild-Type and Transgenic Arabidopsis Plants. The Plant Cell Online. 2003;15:2866–2884.
8. Hegde M, Oliveira JN, Costa JGD, Loza-Reyes E, Bleicher E, Santana AEG, et al. Aphid antixenosis in cotton is activated by the natural plant defence elicitor cis-jasmone. Phytochemistry. 2012;78:81–88.
9. Fontana A, Held M, Fantaye CA, Turlings TC, Degenhardt J, Gershenzon J, et al. Attractiveness of constitutive and herbivore-induced sesquiterpene blends of maize to the parasitic waspcotesia marginiventris (cresson). Journal of Chemical Ecology. 2011;37:582–591.
10. Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, et al. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature. 2005;434:732–737.
11. Xiao Y, Wang Q, Erb M, Turlings TCJ, Ge L, Hu L, et al. Specific herbivore-induced volatiles defend plants and determine insect community composition in the field. Ecology Letters. 2012;15:1130–1139.
12. Clavijo McCormick A, Unsicker SB, Gershenzon J. The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends in Plant Science. 2012;17:303–310.
13. Pichersky E, Noel JP, Dudareva N. Biosynthesis of plant volatiles: nature's diversity and ingenuity. Science. 2006;311:808–811.
14. Unsicker SB, Kunert G, Gershenzon J. Protective perfumes: the role of vegetative volatiles in plant defense against herbivore. Current Opinion in Plant Biology. 2009;12:479–485.
15. Li FQ, Li W, Lin YJ, Pickett JA, Birkett MA, Wu K, et al. Expression of lima bean terpene synthases in rice enhances recruitment of a beneficial enemy of a major rice pest. Plant, Cell & Environment. 2017;41:111–120.
16. Turlings TCJ, Tumlinson JH, Do parasitoids use herbivore-induced plant chemical defenses to locate hosts?. Florida Entomologist. 1991;74:42.
17. Hoballah ME, Degen T, Bergvinson D, Savidan A, Cristina Tamò, Turlings TCJ. Occurrence and direct control potential of parasitoids and predators of the fall armyworm (lepidoptera: noctuidae) on maize in the subtropical lowlands of Mexico. Agricultural & Forest Entomology. 2015;6:83–88.
18. Turlings TCJ, Tumlinson JH, Lewis WJ. Exploitation of Herbivore-Induced Plant Odors by Host-Seeking Parasitic Wasps. Science. 1990;250:1251–1253.
19. Turlings TCJ, Tumlinson JH, Heath RR, Proveaux AT, Doolittle RE. Isolation and identification of allelochemicals that attract the larval parasitoid, cotesia marginiventris (cresson), to the microhabitat of one of its hosts. Journal of Chemical Ecology. 1991;17:2235–2251.
20. Mcgarvey DJ, Croteau R. Terpenoid metabolism. Plant cell. 1995;7:1015–1026.
21. Sandra I, Krause ST, Grit K, Jonathan G, Degenhardt J, Köllner TG. The organ-specific expression of terpene synthase genes contributes to the terpene hydrocarbon composition of chamomile essential oils. BMC Plant Biology. 2012;12:84.
22. Chen F, Tholl D, Bohlmann J, Pichersky E. The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. The

Plant journal. 2011;66:212–229.

23. Brillada C, Nishihara M, Shimoda T, Garms S, Boland W, Maffei ME, et al. Metabolic engineering of the C16 homoterpene TMTT in lotus japonicus through overexpression of (E, E)-geranyllinalool synthase attracts generalist and specialist predators in different manners. New Phytologist. 2013;200:1200–1211.
24. Degenhardt J, Hiltpold I, Tobias GK, Frey M, Gierl A, Gershenzon J, et al. Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proc Natl Acad Sci U S A. 2009;106:13213–13218.
25. Gao L, Zhang X, Zhou F, Chen H, Lin Y. Expression of a peppermint (E)-β-farnesene synthase gene in rice has significant repelling effect on bird cherry-oat aphid (rhopalosiphum padi). Plant Molecular Biology Reporter. 2015;33:1967–1974.
26. Zhang Y, Li ZX, Yu XD, Fan J, Pickett JA, Jones, HD, et al. Molecular characterization of two isoforms of a farnesyl pyrophosphate synthase gene in wheat and their roles in sesquiterpene synthesis and inducible defence against aphid infestation. New Phytologist. 2015;206:1101–1115.
27. Kappers IF. Genetic Engineering of Terpenoid Metabolism Attracts Bodyguards to Arabidopsis. Science. 2005;309:2070–2072.
28. Huang XZ, Xiao YT, Köllner TG, Jing WX, Kou JF, Chen JY, et al. The terpene synthase gene family in\r, gossypium hirsutum\r, harbors a linalool synthase GHTPS12 implicated in direct defense responses against herbivores. Plant, Cell & Environment. 2018;41:261–274.
29. Yuan JS, Köllner TG, Wiggins G, Grant J, Zhao N, Zhuang X, et al. Elucidation of the genomic basis of indirect plant defense against insects. Plant Signaling & Behavior. 2008;3:720–721.
30. Acar EB, Medina JC, Lee ML, Booth GM. Olfactory behavior of convergent lady beetles (coleoptera: coccinellidae) to alarm pheromone of green peach aphid (hemiptera: aphididae). The Canadian Entomologist. 2001;133:9.
31. Cai QN, Zhang QW, Cheo M. Contribution of indole alkaloids to Sitobion avenae (f.) resistance in wheat. Journal of Applied Entomology. 2010;128:517–521.
32. Zadoks JC. Crop production and crop protection: Estimated losses in major food and cash crops. Agricultural Systems. 1994;51:493–495.
33. Fiebig M, Poehling HM, Borgemeister C. Barley yellow dwarf virus, wheat, and sitobion avenae: a case of trilateral interactions. Entomologia Experimentalis Et Applicata. 2010;110:11–21.
34. Quillec LL, Tanguy S, Dedryver CA. Aerial flow of barley yellow dwarf viruses and of their vectors in western France. Annals of Applied Biology. 1995;126:75–90.
35. Rozas Liras JA, Vieira FG. Genome sequence of the pea aphid Acyrthosiphon pisum. Plos Biology. 2010;8:e1000313.
36. Bruce TJA, Aradottir GI, Smart LE, Martin JL, Caulfield JC, Doherty A, et al. The first crop plant genetically engineered to release an insect pheromone for defence. Scientific Reports. 2015;5:11183.

37. Li FQ, Fu NN, Zhou JJ, Wang GR. Functional characterization of (E)-β-caryophyllene synthase from lima bean and its up-regulation by spider mites and alamethicin. Journal of Integrative Agriculture. 2017;16:2231–2238.
38. Richter A, Schaff C, Zhang Z, Lipka AE, Tian F, Köllner TG, et al. Characterization of biosynthetic pathways for the production of the volatile homoterpenes DMNT and TMTT in Zea mays. The Plant Cell. 2016;28:2651–2665.
39. Lee S, Badieyan S, Bevan DR, Herde M, Gatz C, Tholl D. Herbivore-induced and floral homoterpene volatiles are biosynthesized by a single p450 enzyme (CYP82G1) in Arabidopsis [plant_biology]. Proceedings of the National Academy of Sciences of the United States of America. 2010;107:21205–21210.
40. Zebelo S, Song Y, Kloepper JW, Fadamiro H, Rhizobacteria activates (+)-\r, δ\r, -cadinene synthase genes and induces systemic resistance in cotton against beet armyworm (\r, spodoptera exigua\r,). Plant, Cell & Environment, 2016;39:935–943.
41. Williams L 3rd, Rodriguez-Saona C, Paré PW, Crafts-Brandner SJ. The piercing-sucking herbivores Lygus hesperus and Nezara viridula induce volatile emissions in plants. Archives of insect biochemistry and physiology. 2010;58:84–96.
42. Liu D, Huang X, Jing W, An X, Zhang Q, Zhang H, et al. Identification and functional analysis of two P450 enzymes of Gossypium hirsutum involved in DMNT and TMTT biosynthesis. Plant Biotechnology Journal. 2018;16:581–590.
43. Richter A, Seidl-Adams I, Köllner T, Schaff C, Tumlinson, JH, Degenhardt J. A Small, differentially regulated family of farnesyl diphosphate synthases in maize (Zea mays) provides farnesyl diphosphate for the biosynthesis of herbivore-induced sesquiterpenes. Planta. 2015;6:1451–1361.
44. Dahlin I, Vucetic A, Ninkovic V. Changed host plant volatile emissions induced by chemical interaction between unattacked plants reduce aphid plant acceptance with intermorph variation. Journal of Pest Science. 2015;88:249–257.
45. Pichersky E, Noel JP, Dudareva N. Biosynthesis of plant volatiles: nature's diversity and ingenuity. Science. 2006;311:808–811.
46. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. 1995;57:289–300.

Tables

Table 1 Statistical results of the assembled transcripts

Type	Value
Total number	243,718
Total length	203,088,986
N50 length	1041
Mean length	833.2949803
GC%	47.09%

Table 2 The significantly enriched pathways for DEGs in Octoploid Tirtitrigia ears

Term	ID	Input number	P-Value
Ribosome	ko03010	116	1.48E-20
Monoterpenoid biosynthesis	ko00902	15	3.73E-13
Cardiac muscle contraction	ko04260	15	1.94E-05
Alzheimer's disease	ko05010	41	2.00E-05
Huntington's disease	ko05016	46	3.03E-05
Protein processing in endoplasmic reticulum	ko04141	57	3.25E-05

Figures

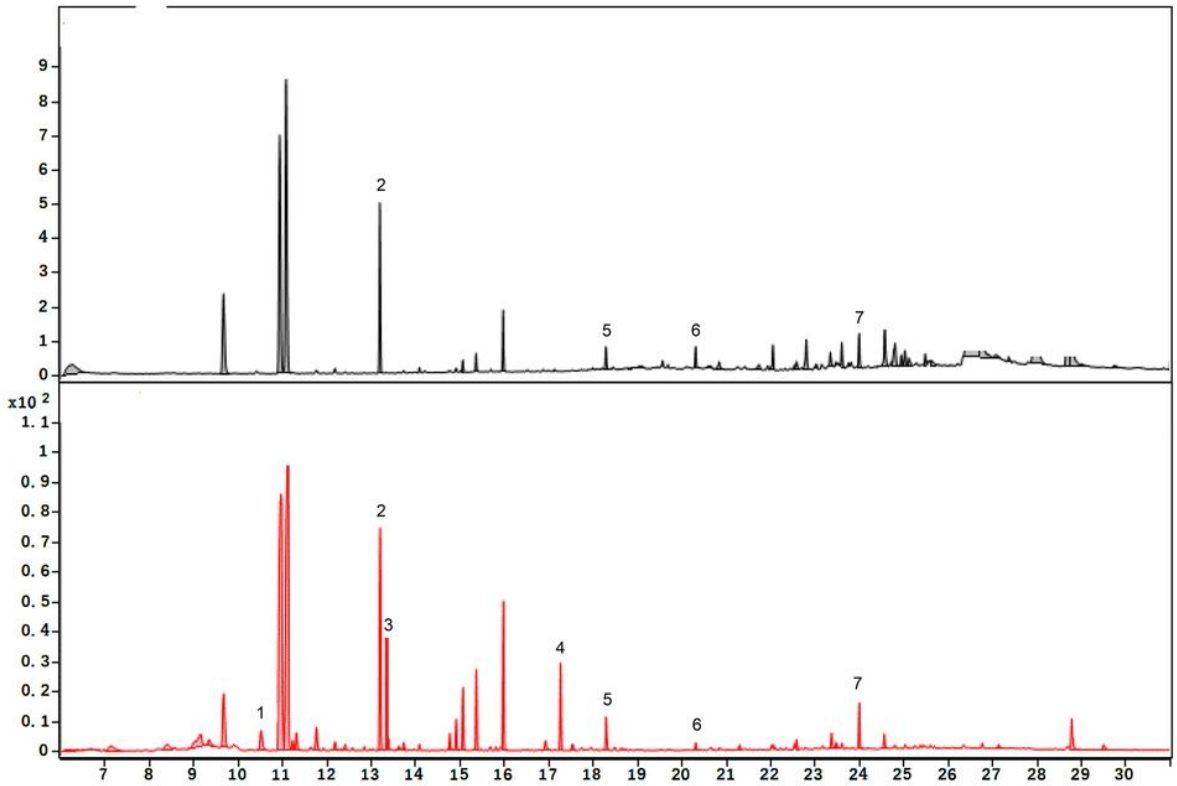

Figure 1

Representative chromatograms of headspace volatile compounds from the intact Octoploid Trititrigia ears and the damaged ears by S. avenae. 1, 2, 3, 4, 5, 6 and 7 refer to β-Myrcene, linalool, DMNT, β-caryophyllene, δ-cadinene, (3S,6E)-nerolidol, and ent-kaurene respectively

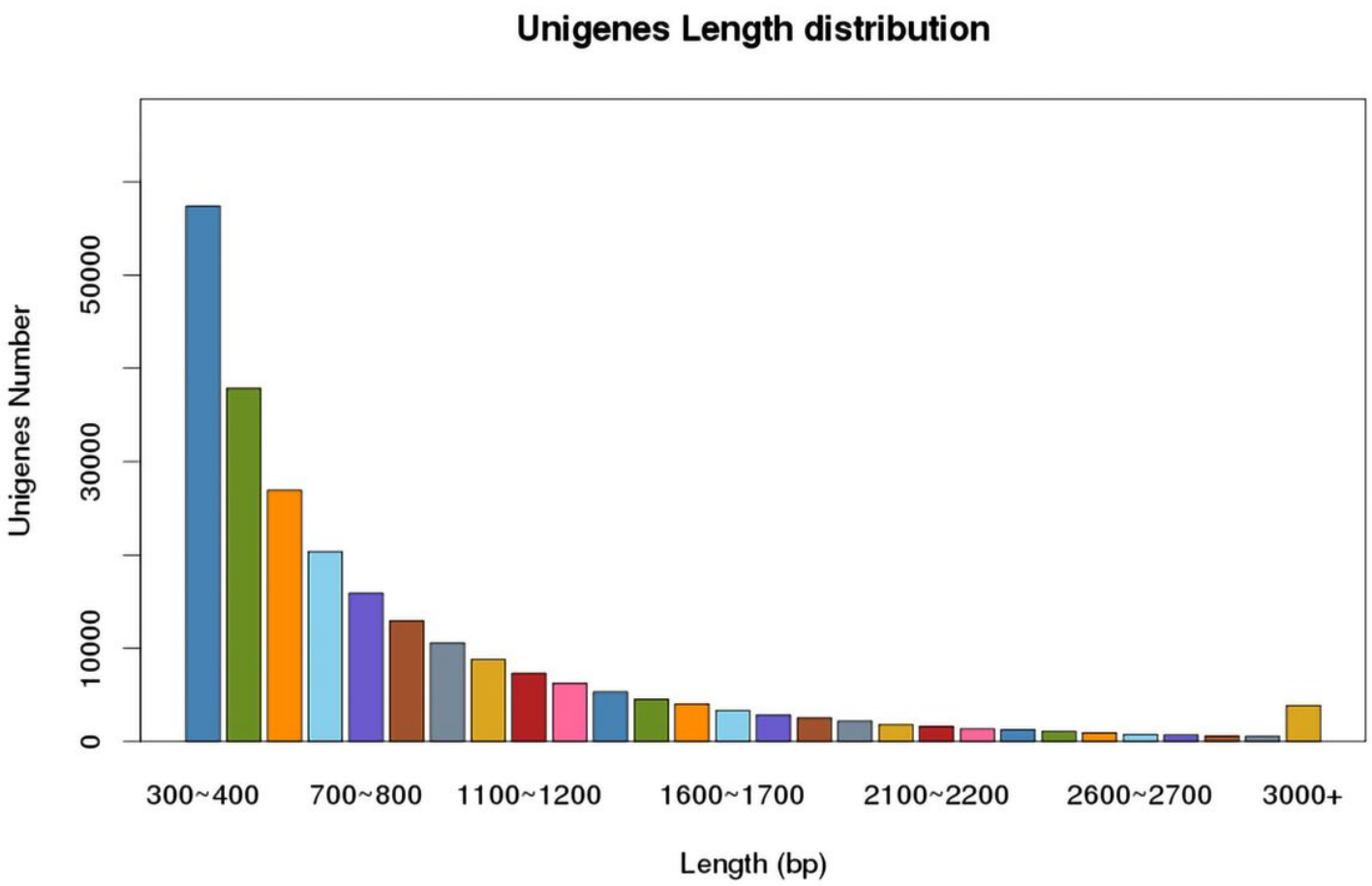

Figure 2

Length distribution of the assembled transcript sequences of Octoploid Trititrigia ears

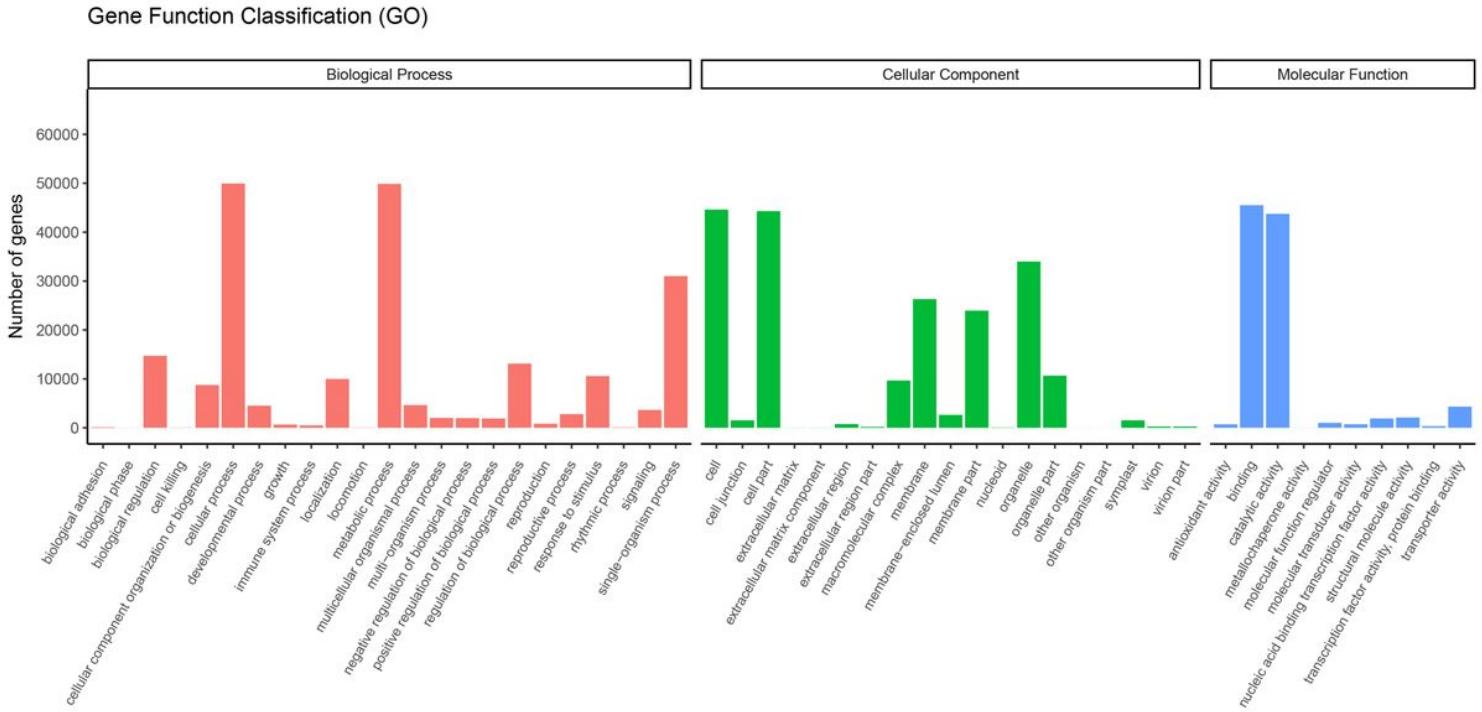

Figure 3

Gene Ontology (GO) classification of Octoploid Trititrigia ears unigenes

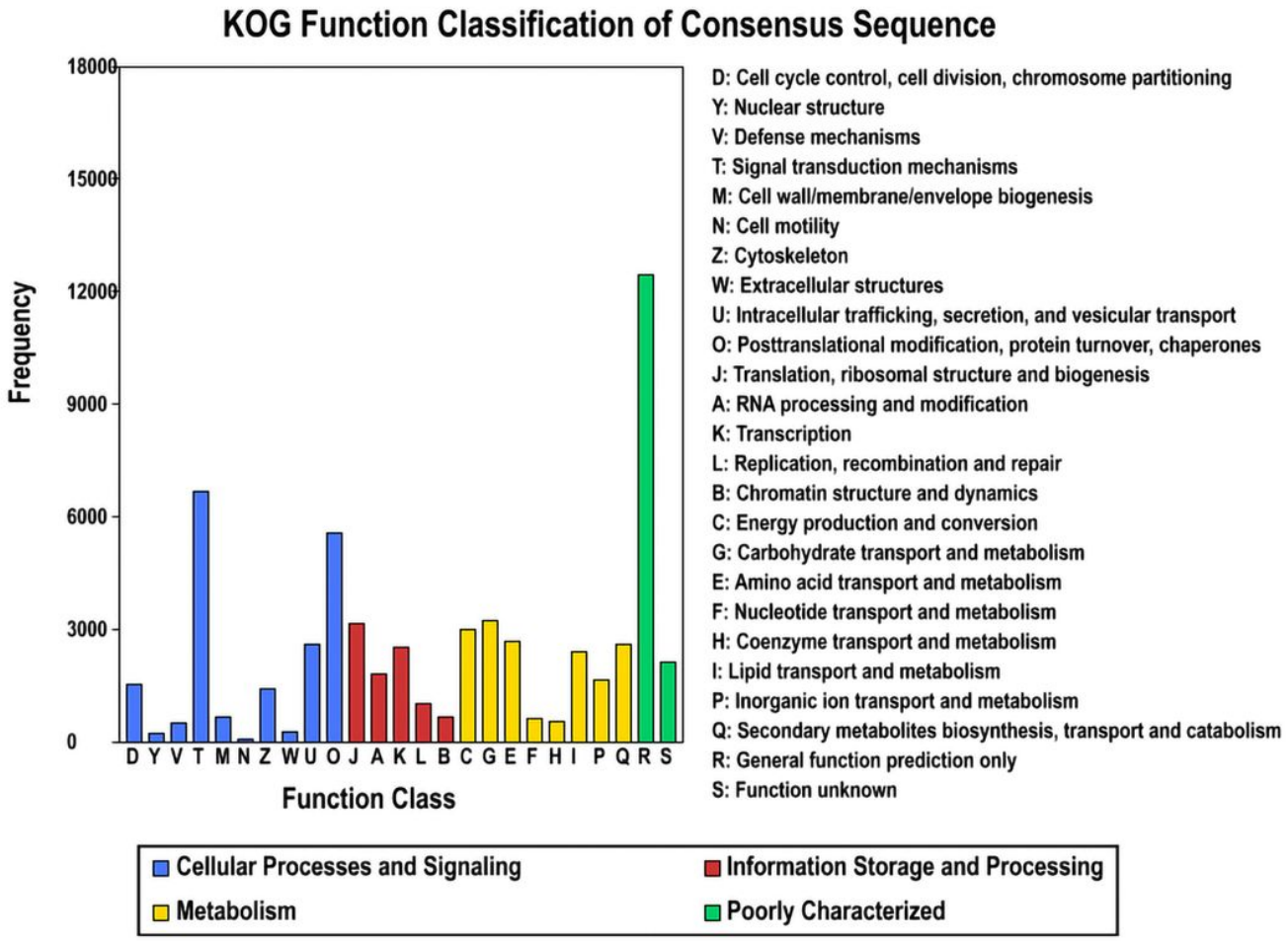

Figure 4

KOG function classification of Octoploid Tirtitrigia ears unigenes

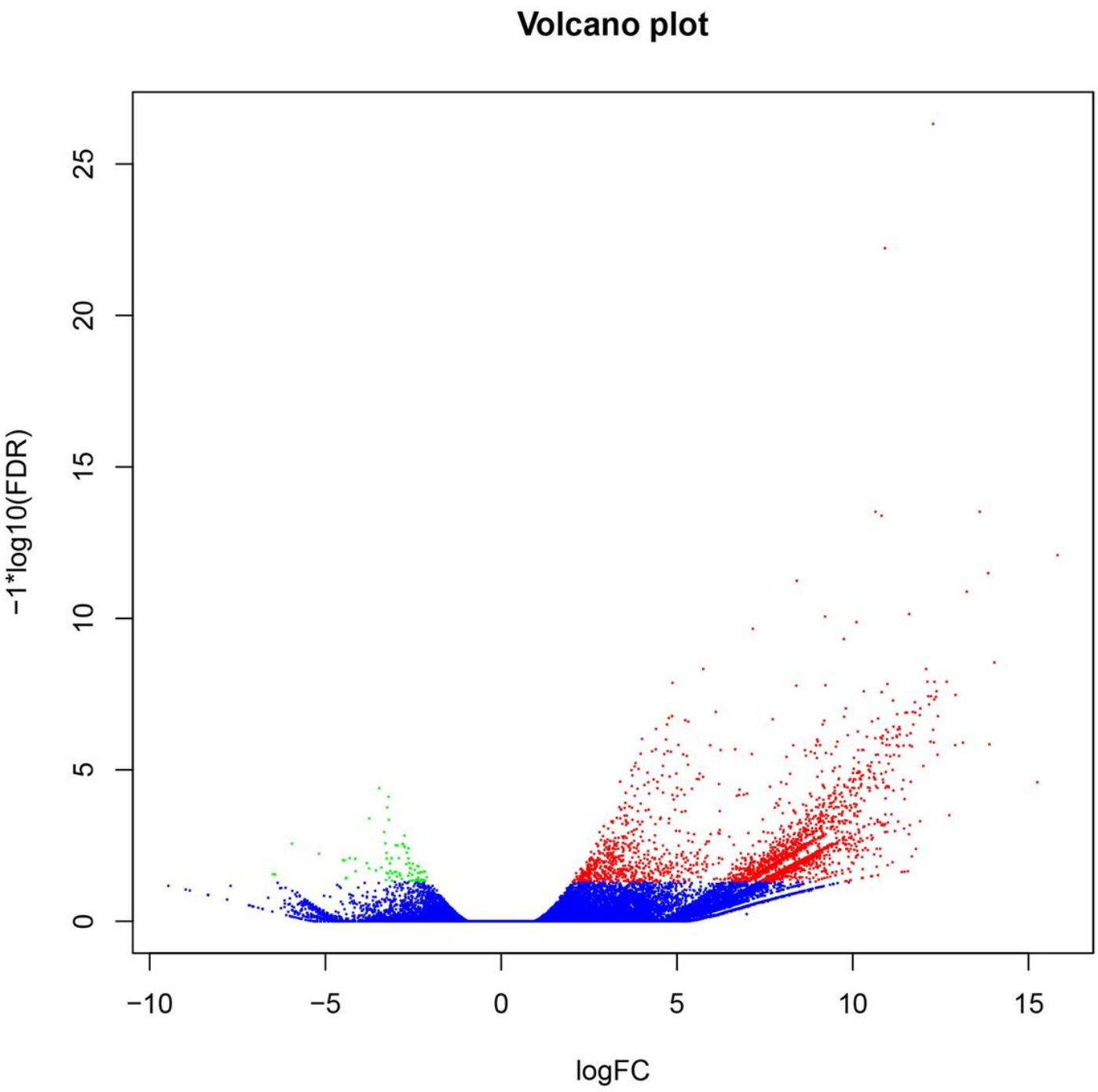

Figure 5

The expression profiles of the identified DEGs. Red and blue points represent the significant DEGs with FDR ≤ 0.05 and log2(fold change) > 1, and green ones show those without significance, respectively. Fold change refers to the values of FPKM change of intact Octoploid Trititrigia vs. aphid damaged Octoploid Trititrigia ear libraries

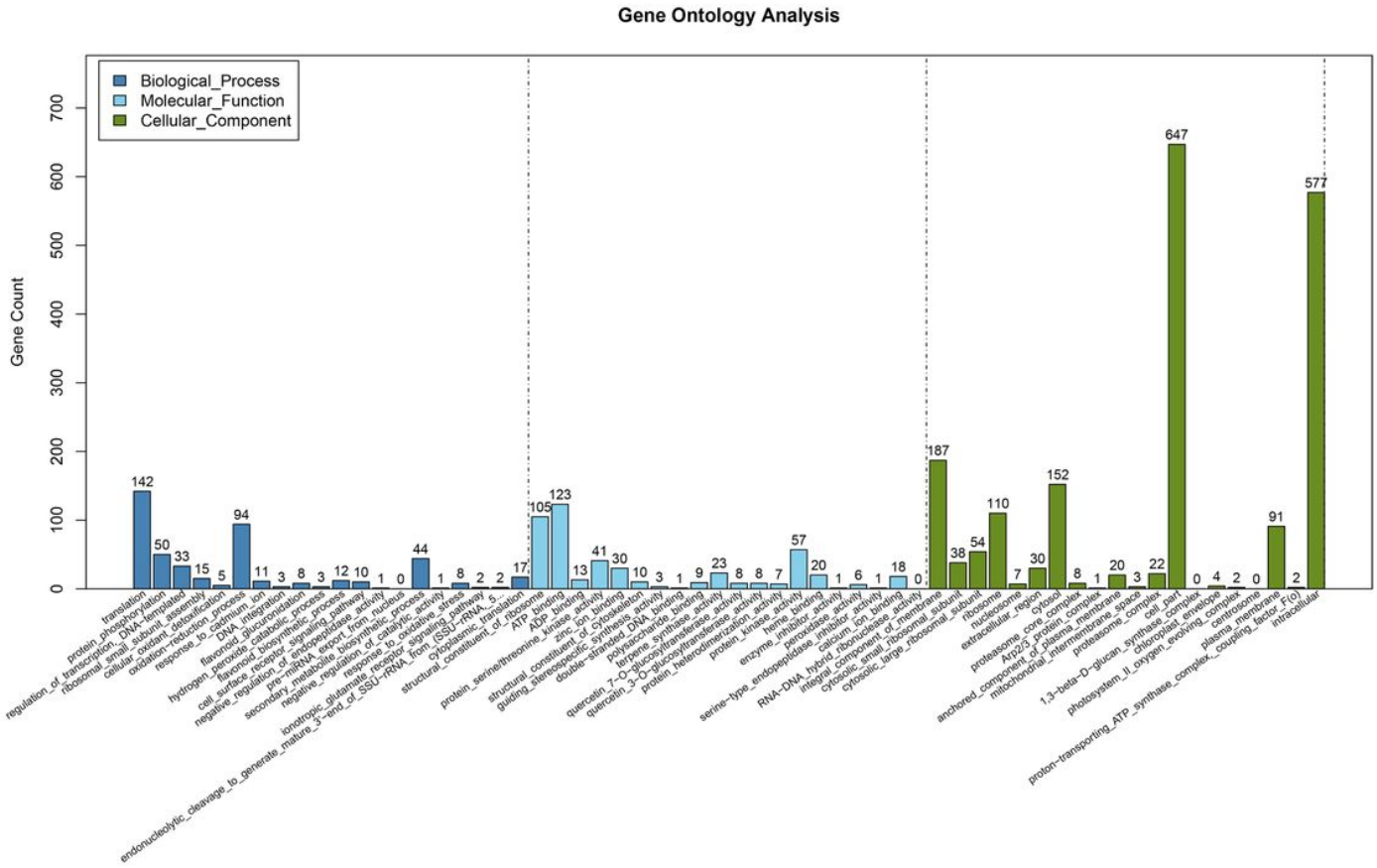

Figure 6

The Gene Ontology (GO) classification of 2389 DEGs. Top 20 GO terms are summarized in three main categories of biological process, molecular function and cellular component

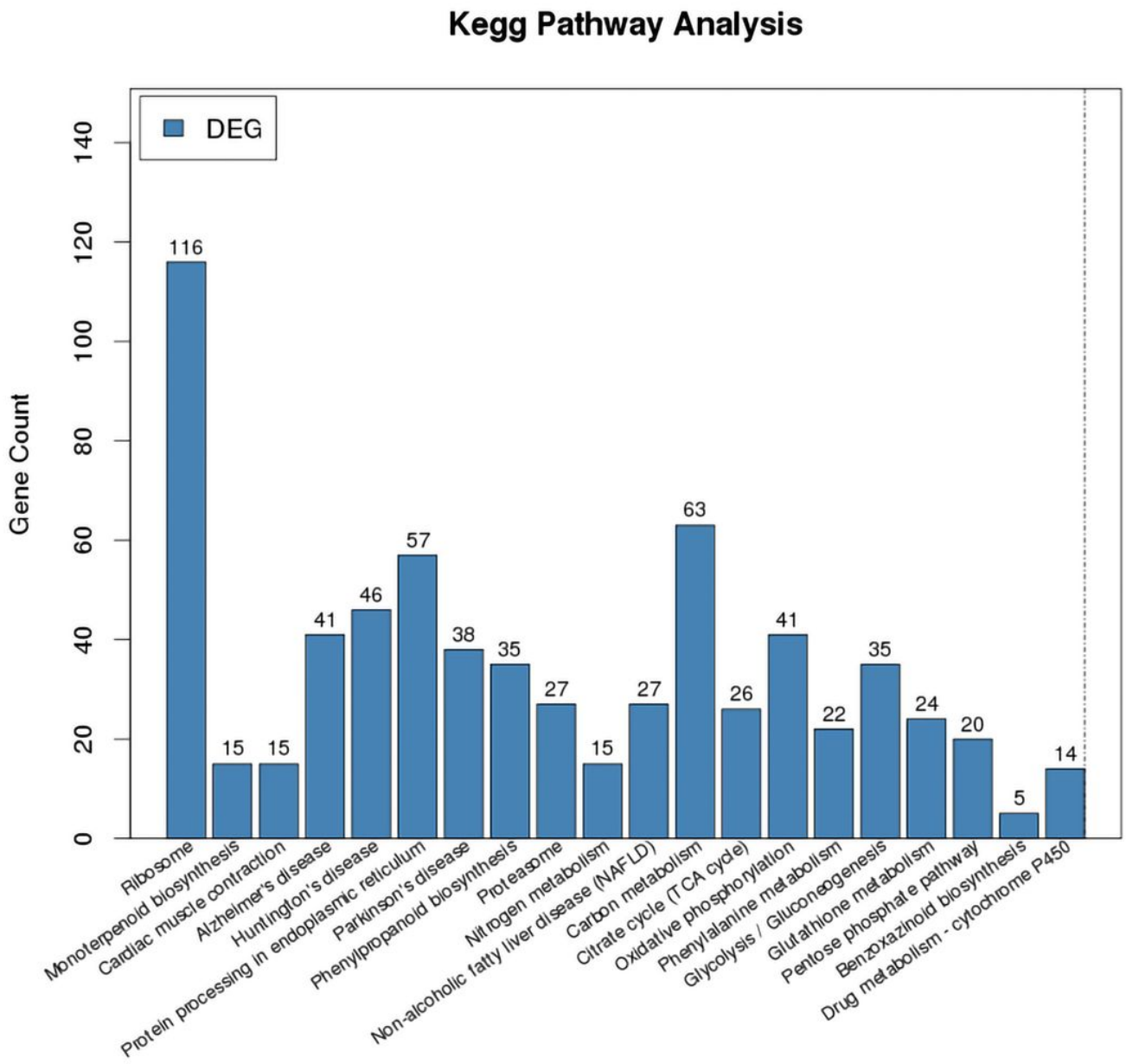

Figure 7

Top 20 pathways of KEGG functional enrichment among DEGs

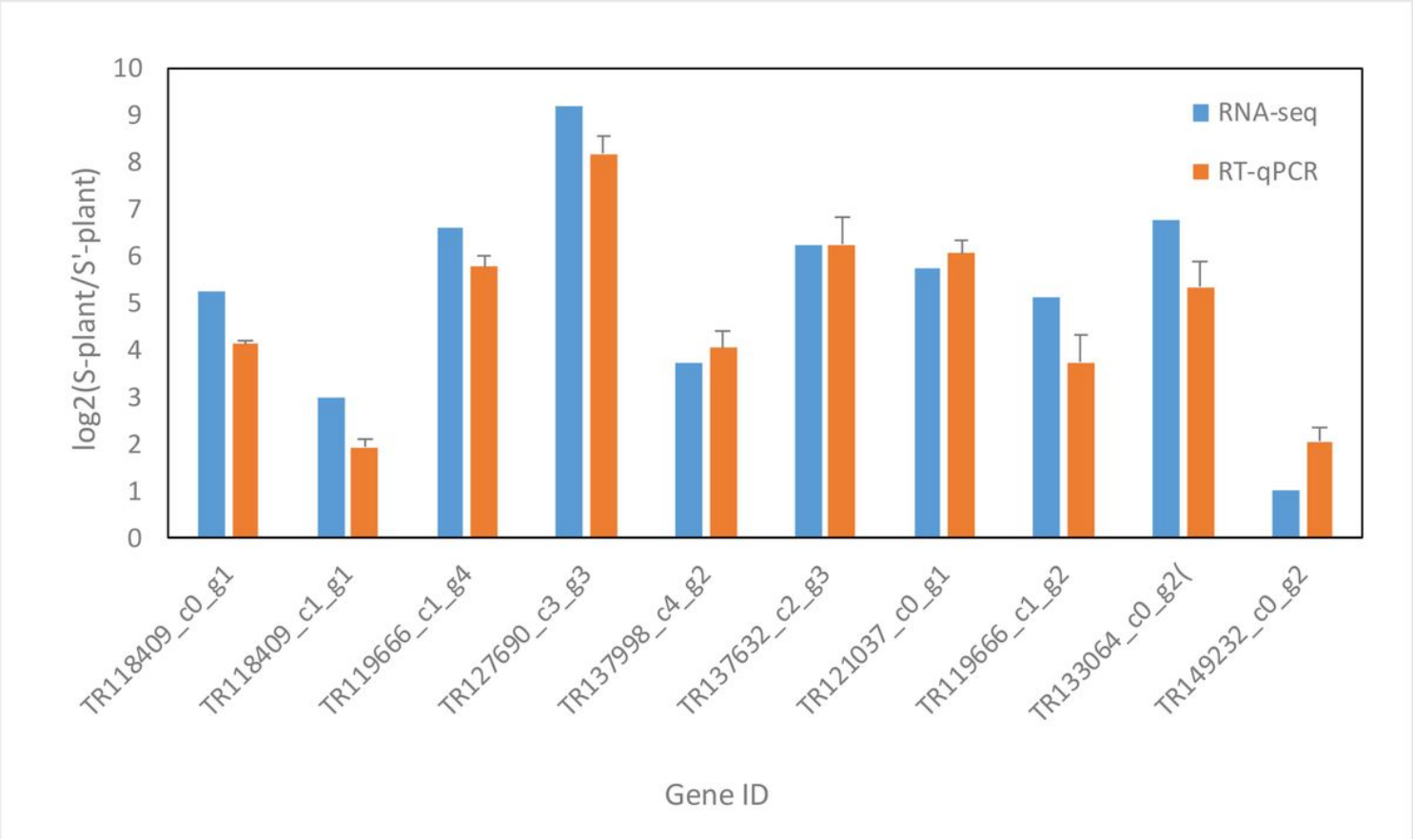

Figure 8

Expression level validation of DEG using RT-qPCR, in comparation to corresponding data detected in RNA-Seq. Relative expression ratio of each DEG is presented in a log2 value of aphid damaged ear vs intact Octoploid Trititrigia ear libraries. The values are mean ± SE (n = 3)

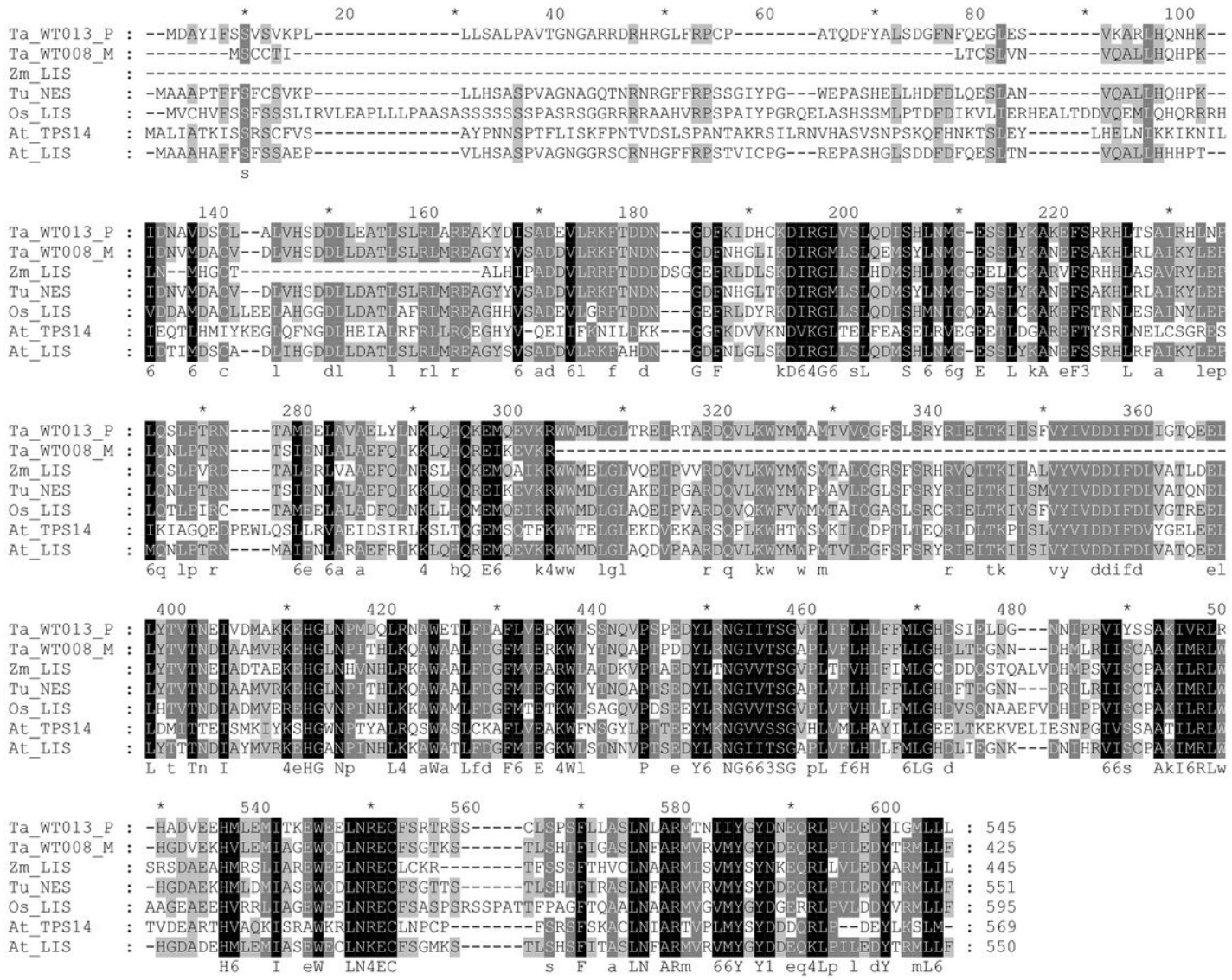

Figure 9

Alignment of TaLIS1 and TaLIS2 Aegilops tauschii LIS (XP_020179676), Oryza sativa LIS (XP_015623808), Zea mays LIS (AQK60700), Triticum Urartu NES1 (EMS50987), Arabidopsis thaliana LIS (NP_176361) amino acid sequences. The highly conserved regions are DDxxD. The alignment was performed by CLUSTALW

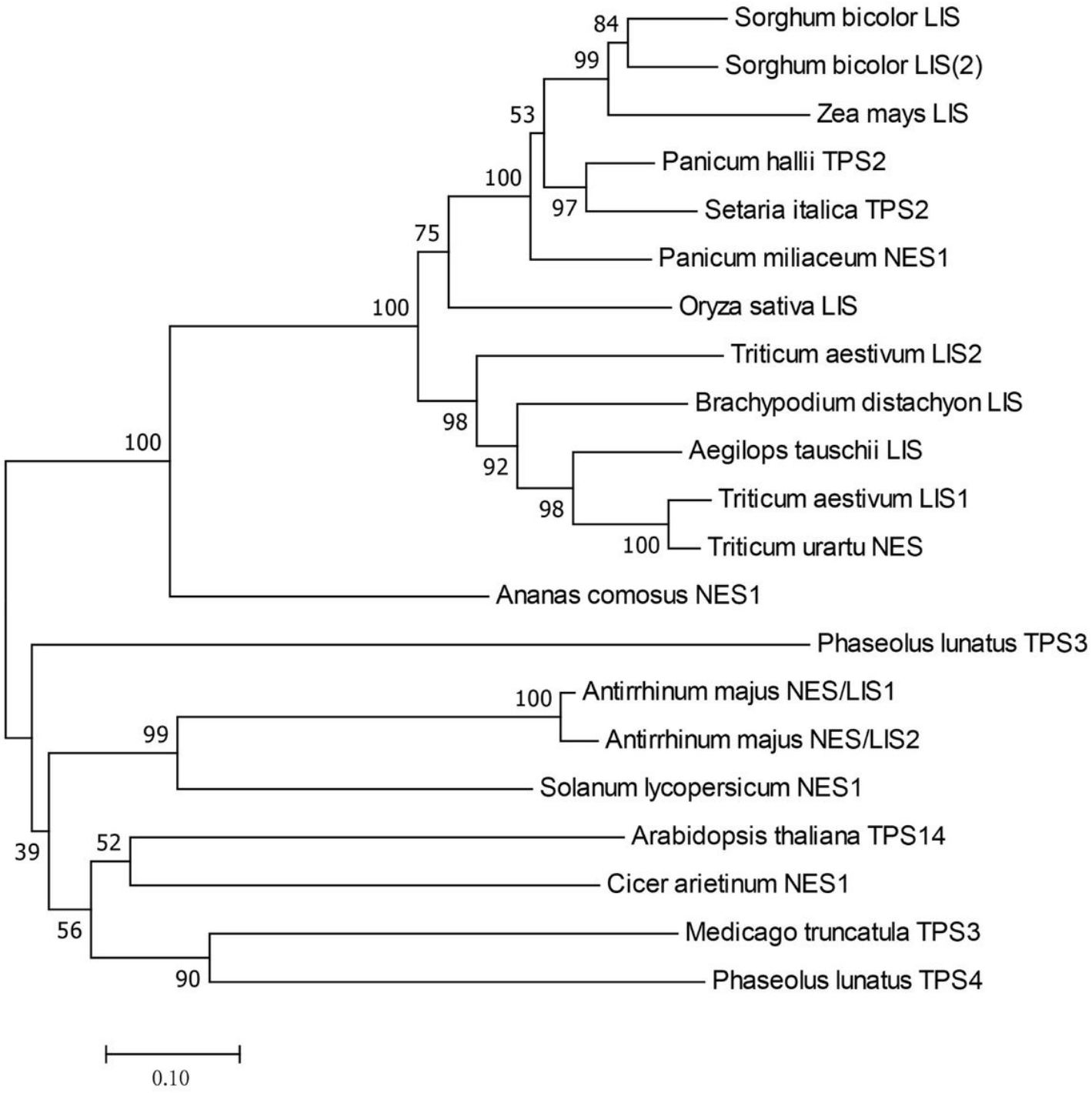

Figure 10

Phylogenetic tree of TaLIS together with known TPSs from other plants. TaLIS fell in the cluster of angiosperm monoterpene synthases (TPS-g). The numbers given at the branches are the bootstrap values. The functional descriptions and accession numbers of these genes were listed in Additional file 8

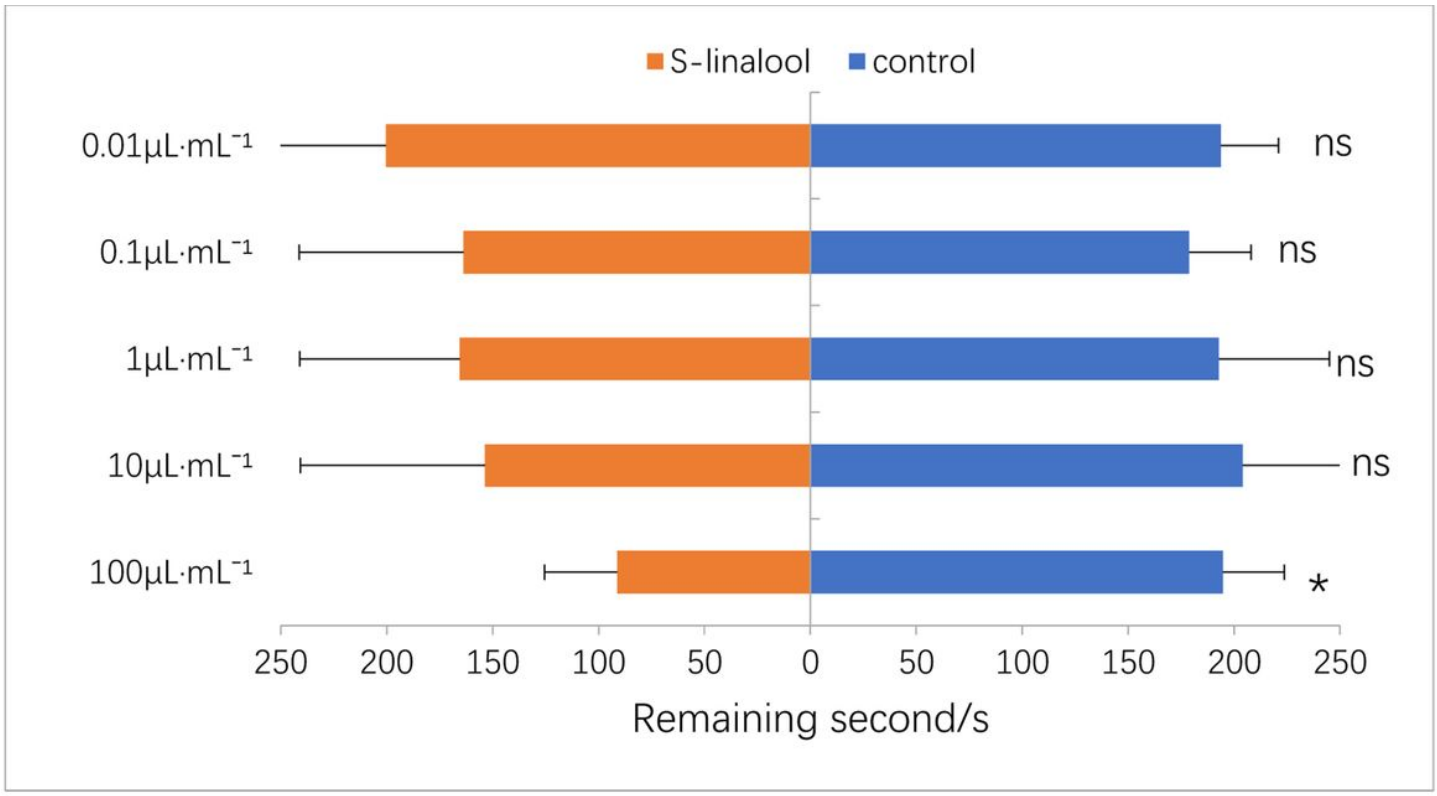

Figure 11

* indicate significant difference (P<0.05) between treatment and control, ns indicates no significant difference based on nonparametric test

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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