De novo assembly and comparative transcriptome analysis revealed the genes that were potentially involved in defensive terpenoids emission in the wheat against the wheat aphid Sitobion avenae (Fabricius)

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Research article

Keywords: Sitobion avenae, Octoploid Trititrigia, Transcriptome, RNA-Seq, Volatile terpenoids, S-linalool

Posted Date: December 6th, 2019

DOI: https://doi.org/10.21203/rs.2.15301/v2

Abstract

Background Terpenoid volatiles play an important role directly or indirectly in the plant defense mechanisms against herbivores, including the gramineous crops such as transgenic rice and corn. The conventional varieties of wheat are important gramineous cereal crops that lack aphid-resistant genes. Therefore, it is necessary to seek the aphid-resistant genes by screening for potential terpenoid synthase genes in the 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 as compared to the 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). After de novo assembly and quantitative assessment, a total of 182348 (74.8%) unigenes were annotated by alignment with the public protein databases. Of these unigenes, 2389 differentially expressed genes were identified between the 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 by showing the significant transcript changes between the intact and damaged plant ears of Octoploid Trititrigia. The transcript abundances of terpenes biosynthetic pathway-related genes were also positively correlated with the production of volatile terpenoids in the 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, the phylogenetic tree results indicated that the TaLIS1 and TuNES1 are highly homologous. Conclusions This assembled transcriptome of S. avenae-damaged Octoploid Trititrigia and the intact ears could provide more molecular resources for the future functional characterization analysis of genomics in volatile terpenoids involved in direct or indirect defenses. Our study describes the metabolic regulation mechanism of the volatile terpenoids in the gramineous crops, which provides support for both breeding and genetic modification of the wheat varieties resistant to wheat aphid.

Background

Plants interact with the environment by producing a variety of chemical compounds. Plants could produce volatile compounds to protect themselves when they are attacked by the herbivorous insects [1, 2]. Herbivore-induced plant volatiles (HIPVs) help plants to protect themselves from the herbivores that might cause mortality or reduced reproductive fitness. HIPVs could deter herbivores in the field [3-5] or the laboratory [6-8] and attract natural enemies of the herbivore [9-12]. Yet, herbivores could also be attracted to the plant volatile compounds induced by the herbivores 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 the 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 damaged by the spider mites, *P. lunatus* leaves release a complex blend of the volatiles, especially the volatile terpenoids which could attract the natural enemy, *P. persimilis* [15]. Studies have shown that in maize (*Zea mays*, Linn.), damaged by lepidopteran larvae such as *Spodoptera littoralis* Boisduval induced 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 the specific terpenoids by using the plants that overexpress the TPS gene [15, 23-27]. Over expression of the wheat FPP synthase gene (*FPS*) in Arabidopsis could repel the *Myzus persicae* Sulzer [26]. Over expression of the *P. lunatus* TPS genes *Pltps3* and *Pltps4* in rice increased the attraction of transgenic rice plants to the natural enemies of the rice stem borer (*Chilo suppressalis* Walker) [15]. The transgenic *Nicotiana tabacum* Linn. plants over expressing the *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 the rice plants damaged by caterpillars of the fall armyworm (*Spodoptera frugiperda* Smith & Abbot) and could significantly attract the parasitic wasps [29]. S-linalool is a key component in the direct and indirect induced volatiles of rice. In the defense responses against *Nilaparvata lugens*, 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 could play a role directly or indirectly in the defense against herbivores.

The English green aphid, *Sitobion avenae* (Fabricius), considered being a one of the most important pests of wheat in Asia and Europe [30-32]. It could cause heavy economic losses to the wheat, both as a phloem feeder and serving as a vector of plant viruses [33-35]. It was reported that the wheat (*Triticum aestivum*, AABBDD) genes *Tafps1* and *Tafps2* played important roles in induced responses to aphid infestation and in sesquiterpene synthesis [26]. The alarm pheromone for many aphid pests, the sesquiterpene (E)-β-farnesene, was successfully developed by the 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 showed considerable potential for the aphid management, but there was no change in aphid numbers in the field trials. This was attributed to low insect numbers and erratic climatic conditions [36]. However, volatiles terpenoids played an important role in direct and indirect plant defense responses against herbivores, especially in the transgenic gramineous crops such as rice and corn [15, 24, 37, 38]. In

addition, the currently reported plant volatiles for wheat aphid control were primarily methyl salicylate [39, 40] and green leaf volatiles [41]. It means that wheat had ability to protect against aphids attack by combining with the volatiles, providing a basis for the study of subsequent volatile terpenes. Moreover, studies have shown that the expression of terpene synthase genes in the plants increased dramatically after pest infestation [15, 42, 43]. Conventional wheat varieties emit few volatile terpenoids which showed resistance to aphids. Due to the long-term directed breeding, the genetic basis of wheat has become narrower and the ability to withstand risks had decreased. Therefore, widening the existing wheat genetic basis, enriching wheat germplasm resources, cultivating high-yield, high-quality and resistant wheat varieties is the current solution.

Thinopyrum intermedium (Host) Barkworth & D.R. Dewey, as a close relative of wheat, has many valuable attributes such as multiflorous, resistant to pests and diseases, and tolerated to the salt and drought. It is one of the most important wild resources that are widely used in the wheat improvement. The octoploid Trititrigia is selected from the cross of the common wheat and *Th.intermedium* (wheat-*Th.intermedium* partial amphiploid). It could comprehend the superior traits of the parents and act as an important bridge parent for transferring the excellent genes of the *Th. intermedium* grass to wheat [44]. Our study revealed that the wheat germplasm resource Octoploid Trititrigia was a promising candidate for breeding aphid-resistant wheat varieties as compared to common wheat. The quality and quantity of the volatile terpenoids changed remarkably in the aphid damaged wheat plants as compared with the intact wheat plants. In order to regulate the synthase genes of the volatile terpenoids which showed resistance to the aphids, we conducted a de novo transcriptome analysis to compare RNA-seq profiles between the 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 the volatile terpenoids, revealing a greatly enriched pathway for the changes of the volatile terpenoids between the 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 intentional. The collected volatile terpenoids were analyzed with transcriptome data. The results provided resources and candidate genes for breeding uniform resistant varieties to wheat aphid.

Results

Emission of the terpenoids from the aphid damaged Octoploid Trititrigia

After Gas Chromatography-Mass Spectrometer (GC-MS) analysis of the volatile compounds, a total ion chromatogram was obtained. The results showed that the aphid damaged Octoploid Tirtitrigia emitted a higher amount of linalool, ent-kaurene, (+)-delta-cadinene and (3S,6E)-nerolidol as compared to non-infested plants. The infested wheat plants emitted new HIPVs including (E)-β-caryophyllene, β-Myrcene and DMNT. (Additional file 1). Additionally, the higher levels of S-linalool were detected in Octoploid Trititrigia ears infested by the *S. avenae* (Fig. 1).

Illumina sequencing and de novo assembly

We used the samples of the Octoploid Trititrigia ear, and produced 42.6 Gb of data. We obtained 243718 sequences after sequence assembly. The total length of the transcribed sequence was 203088986 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 lengths was ranged from 300 to 3000 bp, and the dominate length of the sequences was 300 to 400 bp (Additional file 2). In addition, the sequence length ≥3000bp transcripts numbers were 3863.

Functional annotation and classification

For a comprehensive understanding of the role of the gene of interest, the detected genes were functionally annotated in the databases of NR (NCBI non-redundant protein sequence) public database, Swissport, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Eukaryotic Ortholog Groups (KOG), NT (NCBI nucleotide sequences), Pfam. A total of 182348 (74.8%) unigenes could be annotated into seven databases. Among them, 85145 (34.94%), 42318 (17.36%), 53526 (21.96%), 125347 (51.47%), 138277 (56.74%), 68267 (28.01%) 118398 (48.58%) unigenes were blasted to GO, KEGG, KOG, NR, NT, Swissport and Pfam respectively (Additional file 3).

A Total of 85145 unigenes were assigned to 52 GO terms at the second level (Additional file 4). KOG analysis showed that a total of 53526 non-redundant unigenes with high homology were grouped into 25 functional classes. “General function prediction only” (13453; 25.13%), “Signal transduction mechanisms” (6669; 12.46%), “Posttranslational modification, protein turnover, chaperones” (5573; 10.41%) were the three largest categories (Additional file 5).

Totally 42,318 unigenes were mapped onto the 133 KEGG pathway, and the most enriched sequences were “Ribosome” (1749; 4.13%), followed by “Carbon metabolism” (1738; 4.11%), “Biosynthesis of amino acids” (1490; 3.52%), and “Plant-pathogen interaction” (1334; 3.15%) (Additional file 6).

Identification and functional enrichment analysis of DEG

Based on the FPKM method, the transcript abundance of each gene from the intact Octoploid Trititrigia ears and the aphid-damaged ears were analyzed. A total of 233023 DEGs were identified between the intact and damaged Octoploid Trititrigia ear libraries (Additional file 7). Out of these DEGs, 2389 genes exhibited a significant difference in their expression levels with the threshold of FDR ≤ 0.05, including 2306 up-regulated and 83 down-regulated ones (Fig. 2, Additional file 8). 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 (Additional file 9,). The terms of “Structural_constituent_of_ribosome” (GO:0003735), “ATP_binding”, (GO:0005524) were the dominant groups in the molecular functions; “Cell_part” (GO:0044464), “Intracellular” (GO:0005622) were the representative groups in the cellular components. Among the biological processes, a great number of DEGs were categorized under and oxidation-reduction process (GO:0055114).

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 10), the most significant terpenoid KEGG pathways (p-value ≤ 0.05) consisted of “Terpenoid backbone biosynthesis” (ko00900) “Monoterpenoid biosynthesis” (ko00902), “Diterpenoid biosynthesis” (ko00904), “Carotenoid biosynthesis” (ko00906), “Steroid biosynthesis” (ko00100).

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 the intact and aphid damaged ears of Octoploid Trititrigia (Additional file 11). 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 using RT-qPCR. The levels of genes encoding TPSs, including the genes of annotation in databases encoding Myrcene synthases, cytochrome P450 oxidase, (3S,6E)-nerolidol synthases, 3S-linalool synthases and (+)-delta-cadinene synthases, were significantly higher in the aphid damaged Octoploid Trititrigia than those of intact Octoploid Trititrigia ears (Fig. 3).

Sequence analysis of the 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 the 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 an 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 the typical

conserved motifs and domains of the specific terpene synthase (Fig. 4). *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 gene was clustered with angiosperm monoterpene synthases (TPS-g family) based on the phylogenetic relationship analysis (Fig.5). 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 had a close 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 to different doses of S-linalool were investigated. The results indicated that when S-linalool concentration was 100μL·mL^{-1}, it had a significant repellent effect on aphids ($W = -3.385$, $P < 0.05$) (Fig. 6).

Discussion

The results of this study revealed that the *S. avenae* could induce biosynthesis of the volatile terpenoids such as S-linalool, DMNT, (E)-β-caryophyllene with significant ramifications for the insect-plant interactions. Specifically, higher levels of S-linalool were detected in Octoploid Trititrigia ears infested by the *S. avenae* in comparison with the uninfested ears. Previous studies have shown that S-linalool was the most abundant volatile emitted from the pest-damaged rice and cotton plants and showed direct or indirect role in defense against herbivores insect pests [28, 29]. Also, *A. thaliana* plants genetically engineered to release S-linalool, repelled the aphids [7]. Additionally, the field studies conducted with *OsLIS*-silenced rice plants showed that inducible (3S)-linalool attracted the predators, parasitoids as well as chewing herbivores, but repelled the rice *N. lugens* [11]. Although the olfactory response of *S. avenae* indicated that only higher concentration of S-linalool (100 μL·mL^{-1}) had remarkable repellent effect on *S. avenae*, it was reported that the mixing of various terpenoids had a significant avoidance effect on *S. avenae* [45]. This provides a new idea for the development of sustained release agents for the terpenoids that avoid the aphids in the field. 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 the *S. avenae*-damaged plant ears than the intact ears ones. Likewise, the infested ears emitted new HIPVs such as β-Myrcene, (E)-β-caryophyllene and DMNT. Previous studies have shown that DMNT, (E)-β-caryophyllene, (+)-delta-cadinene could act as a direct or indirect defense substances against the pests on the plants [11, 23, 37, 38]. The functional significances of the other volatile terpenoids to the 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, 46]. Many structural genes involved in TPSs have been discovered in the field crops in the past few years [15, 36, 47, 48], which had greatly promoted the 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 mechanisms in the plants [15, 28, 38]. The emission of the volatile terpenoids were higher in *S. avenae*-treated Octoploid Trititrigia ears than untreated ears. However, little has been known about the molecular mechanisms that controlling the TPSs biosynthesis in the wheat. Thus, an ample identification of DEGs and modulating pathways related to the TPSs was profiled using de novo transcriptome data via comparison between intact and the aphid damaged Octoploid Trititrigia ears. In addition, the number of genes up-regulated in DEGs was much larger than that of down-regulated genes. It is speculated that after the octoploid plants were infested by S. avenae, A series of defense signals are activated. Thereby inducing the expression of defense genes, resulting in a much higher number of up-regulated genes than down-regulated genes.

TPSs and their volatile compounds, volatile terpenoids, play an important role in direct and indirect plant defense responses against herbivores [10, 49, 50]. 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 the intact Octoploid Trititrigia plant ears. Based on RT-qPCR results, the expression profiles of the 10 genes were identical by the 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 the aphid-damaged Octoploid Trititrigia tissues, which be accorded with previous findings about changes of volatile terpenoids in other plants after pest infestation [27]. Such information could help to provide a deeper understanding of how changes in the gene expression are related to the volatile terpenoids changes in the Octoploid Trititrigia ears.

The GC-MS analysis of the volatile terpenes was combined with transcriptome data. The results showed that the quality and quantity of the terpenoids volatile compounds in *S. avenae* damaged ear changed as 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 could be mapped to the cloned cDNA WT008_M07 (AK333728) and WT013_P07 (AK335856) of Chinese spring wheat cultivar, and the predicted protein sequence could be compared with the S-linalool synthase gene of other species. Phylogenetic tree results confirmed that the *TaLIS1* and *TuNES1* were highly homologous. TPSs are multifunctional enzyme [23]. S-linalool synthase genes at the same time also could 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 could be used as a database of the 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 revealed that the quality and quantity of volatile terpenoids from Octoploid Trititrigia varied dramatically after aphid damage. The transcriptome dataset of Octoploid Trititrigia showed that 243718 unigenes were totally identified and annotated 182348 unigenes (74.8%). Meanwhile, 2389 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 the *S. avenae* infestation under different metabolic processes. This would enable the genetic manipulation of Octoploid Trititrigia to strengthen the aphid resistance.

Materials And Methods

Insects and plants

The colony of wheat aphid, *S. avenae*, were reared on seedlings of the wheat lumai 21 in an environmental chamber (21℃, 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 breed by introduction of the genome of *T. intermedium* into common wheat. Octoploid Tirtitrigia was provided by Dr. Xinfeng Li from National Wheat Improvement Center of China. We declared that the collection of the 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 onto 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), opened 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, by using seal to 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 the 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 500μL 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 done by electron impact (70 eV) and the scanned 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 by 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 by 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, SwissProt, KEGG and KOG, Pfam and GO databases, respectively. The best hit of alignment was used to infer biological function of assembled transcripts. Additionally, GO 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 by using WEGO software for all the transcripts. KEGG pathway annotations were retrieved from the 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 by using the 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 (DEGs) 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 [51].

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 13). The qPCR used Top Green qPCR SuperMix (TransScript) in 20μL reactions according to the manufacturer's instructions. qPCR was performed by using the LightCycler96 Real time PCR System. 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 pI and molecular Mw of the Octoploid Trititrigia S-linalool synthase gene were 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 the phylogenetic relationship, the neighbour-joining (NJ) tree was conducted using MEGA7.0 and 1000 replicates of bootstrap analyses.

Four-arm olfactometer assays

Four-arm olfactometer was 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; DMNT: (E)-4,8-dimethyl-1,3,7-nonatriene; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; GGPP: Geranyl diphosphate; DEGs: Differentially expressed genes; 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; Mw: molecular weight; pI: isoelectric point

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 analyzed 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 to research period but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Key R&D Program of China (2017YFD0201705), and the Funds of Shandong ‘Double Tops’ Program (SYL2017XTTD11). The funding agent only provided the financial support and did not involve in the design of the experiment, collection, interpretation and analysis of data and in the drafting of the manuscript.

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.

Acknowledgements

We would like to thank Dr. Xinfeng Li from National Wheat Improvement Center of China to provide wheat germplasm resources.

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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. 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. 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. 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. Wang G, Cui LL, Dong J, Francis F, Liu Y, Tooker JF. Combining intercropping with semiochemical releases: optimization of alternative control of Sitobion avenae in wheat crops in China. Entomologia Experimentalis Et Applicata. 2011;140:189-195.
40. Wang K, Liu JH, Zhan YD, Liu Y. A new slow-release formulation of methyl salicylate optimizes the alternative control of Sitobion avenae (Fabricius) (Hemiptera: Aphididae) in wheat fields. Pest Management Science. 2019;75:676-682.
41. Visser JH, Yan FS. Electroantennogram responses of the grain aphids *Sitobion avenae* (F.) and *Metopolophiurn dirhodurn* (Walk.) (Horn., Aphididae) to plant odour components. Journal of Applied Entomology. 1995;119:539-542.
42. 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.
43. 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.
44. Gupta PK. Use of Alien Genetic Variation for Wheat Improvement. Springer International Publishing. 2016.
45. Bruce TJ, Birkett MA, Blande J, Hooper AM, Martin JL, Khambay B, et al. Response of economically important aphids to components of Hemizygia petiolata essential oil. Pest Management Science. 2005; 61:1115-1121.
46. 46. 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.
47. 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.
48. 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.
49. 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.
50. Pichersky E, Noel JP, Dudareva N. Biosynthesis of plant volatiles: nature's diversity and ingenuity. Science. 2006;311:808–811.
51. 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%

Additional File Legends

Additional file 1: Representative chromatograms of headspace volatile compounds from the intact Octoploid Trititrigia ears and the damaged ears by *S. avenae*. β-Myrcene, linalool, DMNT, β-caryophyllene, δ-cadinene, (3S,6E)-nerolidol, and ent-kaurene respectively

Additional file 2: Length distribution of the assembled transcript sequences of Octoploid Trititrigia ears

Additional file 3: The information of genes annotation to seven databases

Additional file 4: Gene Ontology (GO) classification of the Octoploid Trititrigia ears unigenes

Additional file 5: KOG function classification of the Octoploid Tirtitrigia ears unigenes

Additional file 6: KEGG pathway analysis of annotated unigenes of the Octoploid Tirtitrigia ears

Additional file 7: All the identified DEGs in the intact and damaged plant ears of the Octoploid Trititrigia libraries

Additional file 8: Differentially expressed unigenes of the intact and damaged plant ears of the Octoploid Trititrigia libraries

Additional file 9: 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

Additional file 10: KEGG enrichment analysis of differentially expressed genes in the Octoploid Tirtitrigia ear

Additional file 11: The key structure genes encoding enzymes to TPSs were detected between the intact plant ears and damaged plant ears

Additional file 12: Protein sequences used for phylogenetic analysis of the plant TPSs

Additional file 13: Primers used in the quantitative real-time PCR

Figures

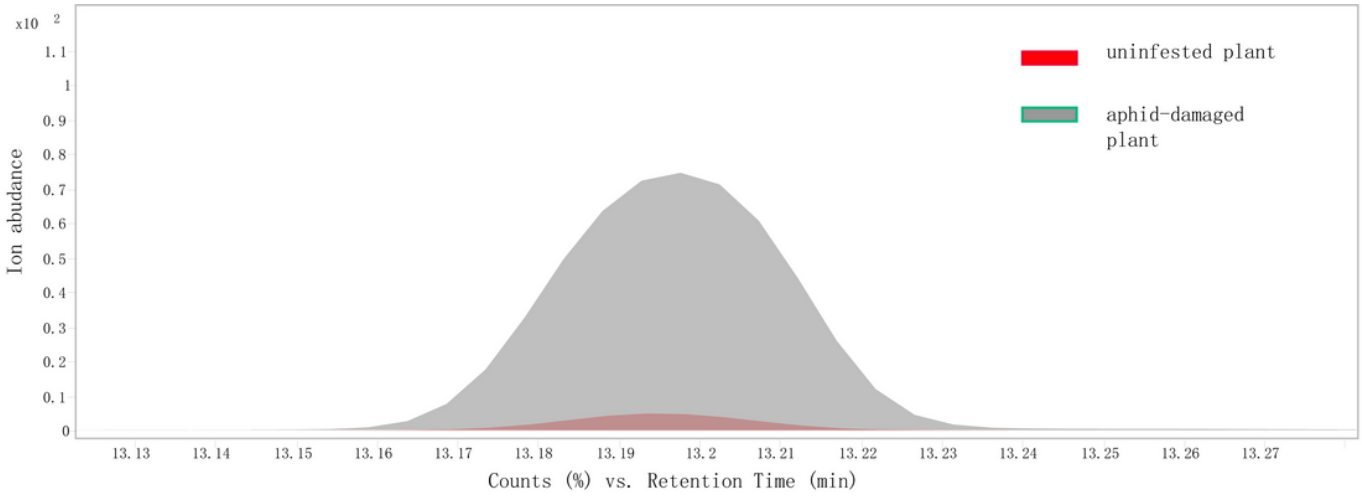

Figure 1

The peak area of headspace volatile compounds Linalool from the damaged ears by S. avenae compared to intact Octoploid Trititrigia ears

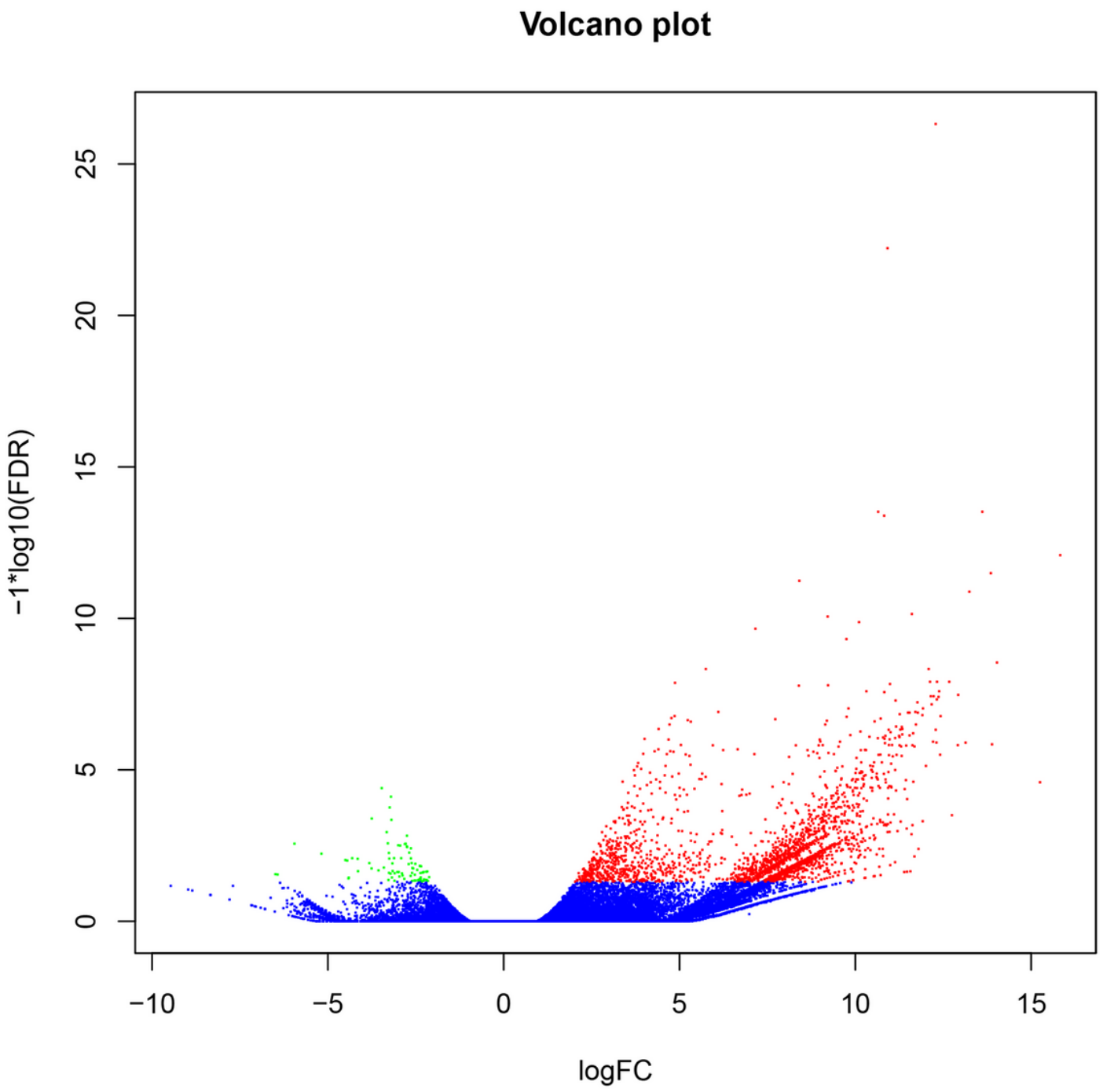

Figure 2

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 the intact Octoploid Trititrigia vs. the aphid damaged Octoploid Trititrigia ear libraries

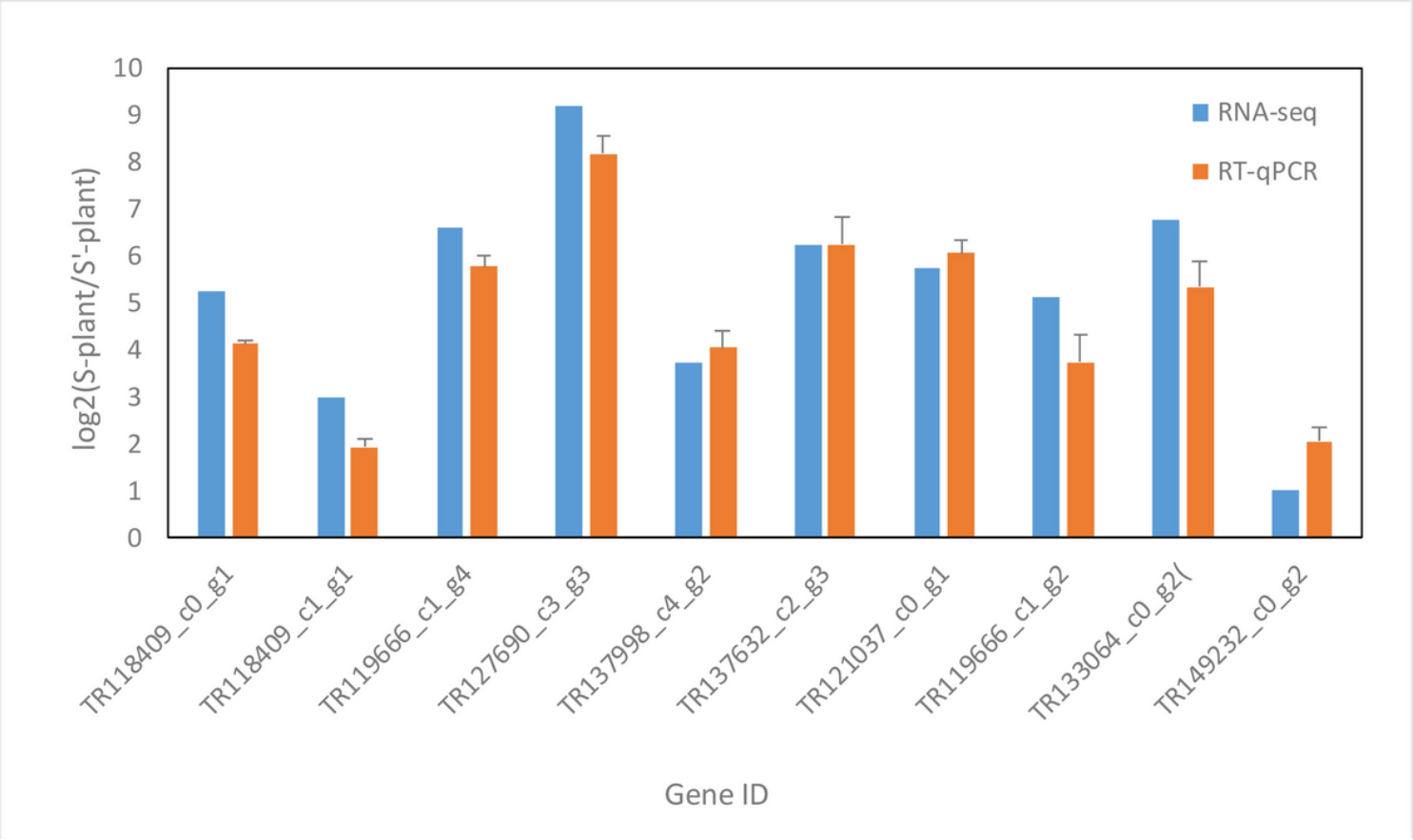

Figure 3

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

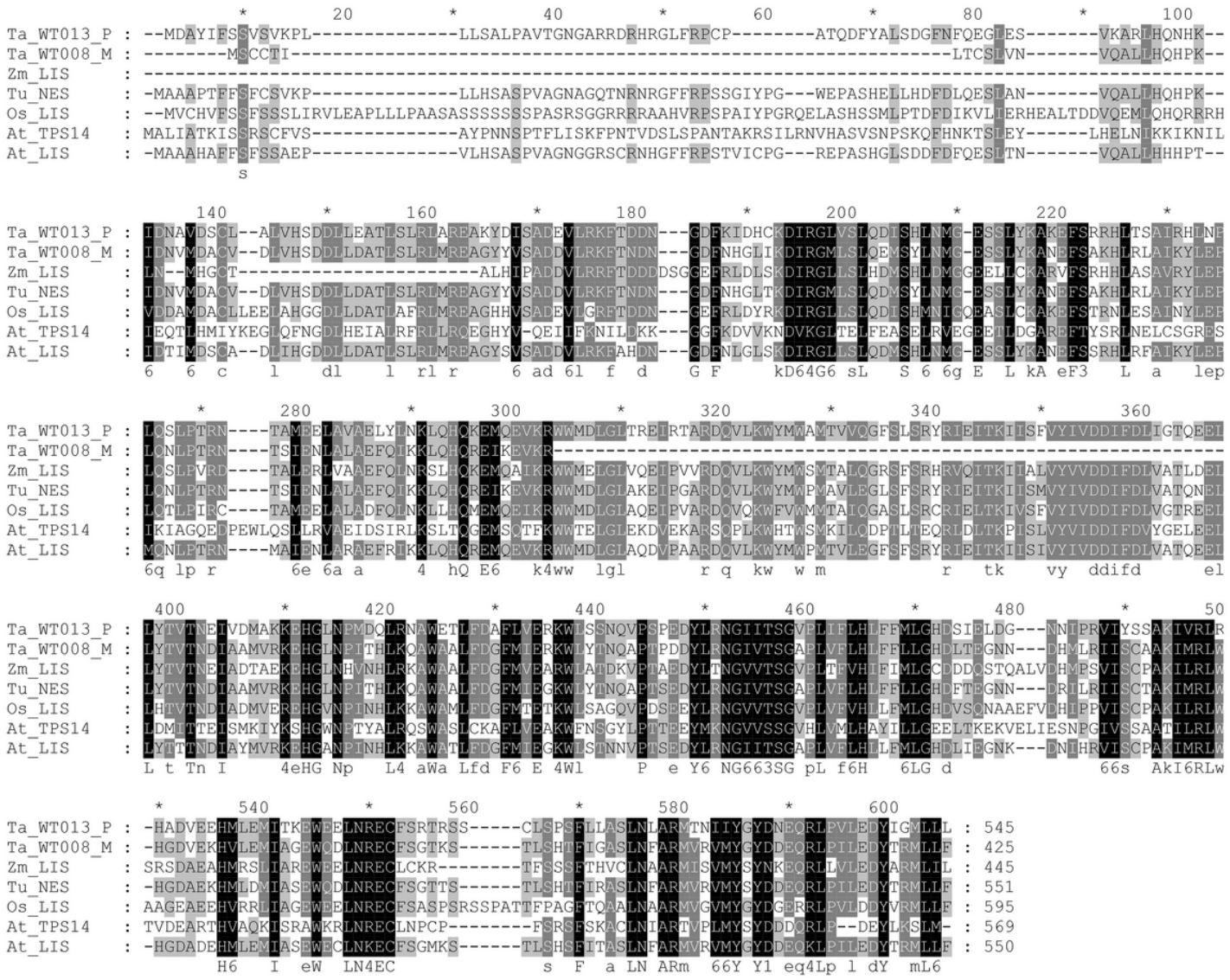

Figure 4

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 were performed by CLUSTALW

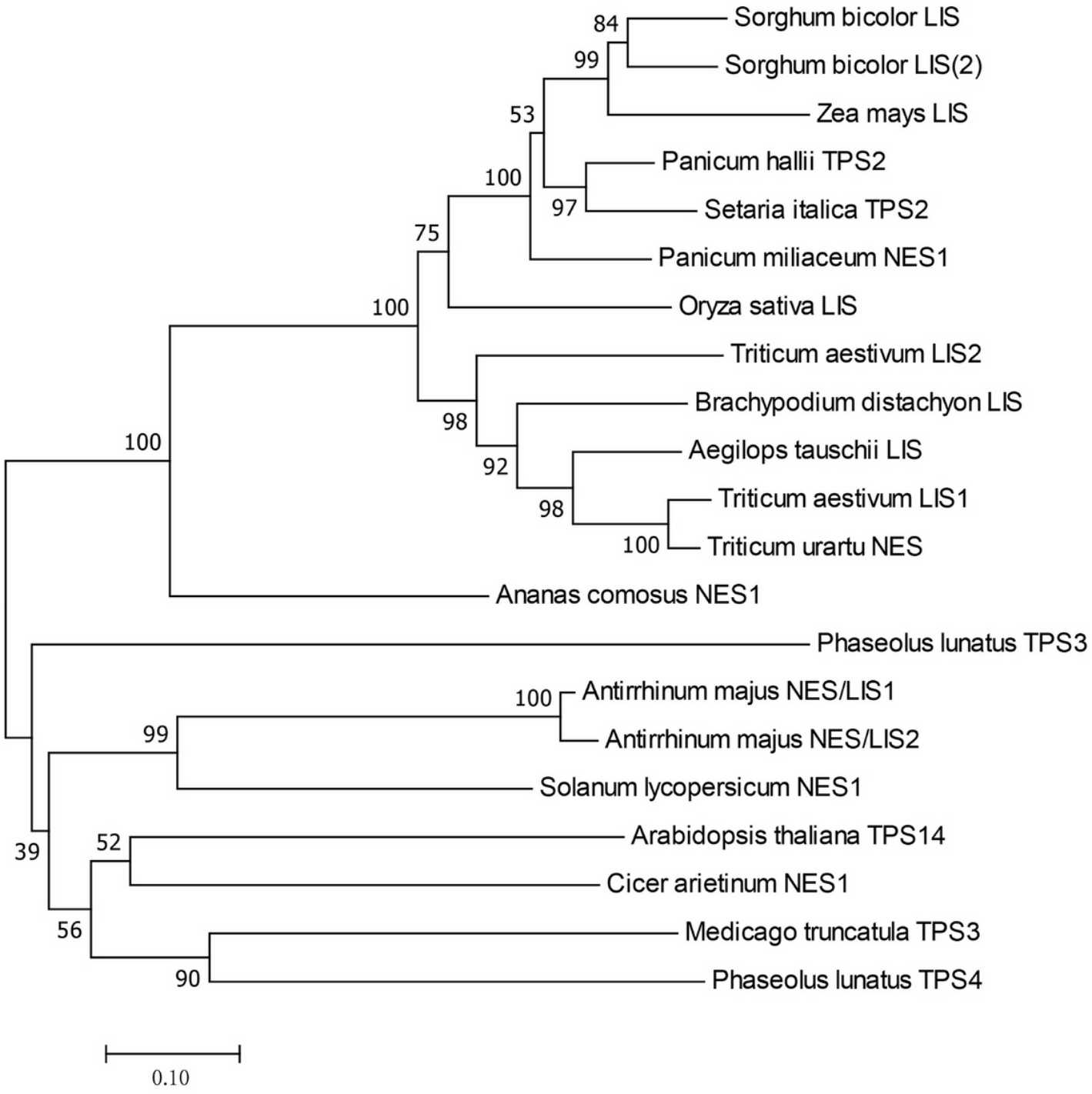

Figure 5

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 12

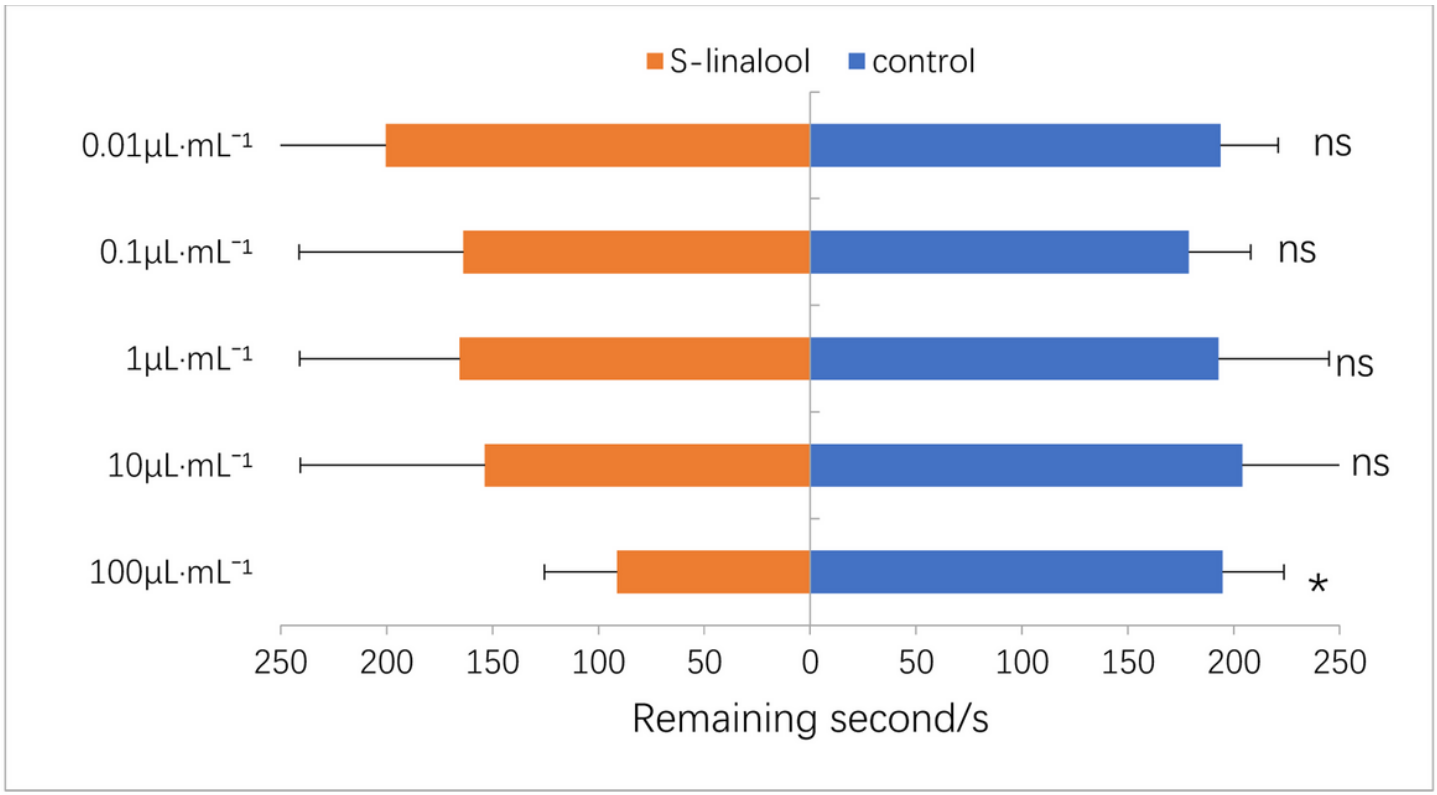

Figure 6

Behavioral response of the S. avenae exposed to S-linalool at different mass concentrations. * 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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