

Terpene Synthase Gene *OtLIS* Confers Wheat Resistance to *Sitobion avenae* by Regulating Linalool Emission

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ABSTRACT: *Sitobion avenae* (Fabricius) is a major insect pest of wheat worldwide that reduces crop yield and quality annually. Few germplasm resources with resistant genes to aphids have been identified and characterized. Here, octoploid *Triticum aestivum*, a species used in wheat distant hybridization breeding, was found to be repellent to *S. avenae* after 2 year field investigations and associated with physiological and behavioral assays. Linalool monoterpene was identified to accumulate dominantly in plants in response to *S. avenae* infestation. We cloned the resistance gene *OtLIS* by assembling the transcriptome of aphid-infested or healthy octoploid *Triticum aestivum*. Functional characterization analysis indicated that *OtLIS* encoded a terpene synthase and conferred resistance to *S. avenae* by linalool emission before and after aphid feeding. Our study suggests that the octoploid *Triticum aestivum* with the aphid-resistant gene *OtLIS* may have potential as a target resource for further breeding aphid-resistant wheat cultivars.

KEYWORDS: aphid resistance, gas chromatography-mass spectrometer, octoploid *Triticum aestivum*, RNA-seq, S-linalool synthase, volatile terpenoids

INTRODUCTION

Outbreaks of the grain aphid, *Sitobion avenae* (Fabricius), on the wheat (*Triticum aestivum* L.) filling stage in a temperate zone threaten wheat production and food safety.^{1–4} *S. avenae* causes significant yield losses in cereals worldwide by phloem sap-sucking, excreting honeydew, and transmitting plant viruses.^{5–8} Aphid control in cereal production necessitates routine insecticide use with undesirable environmental effects and an increase in pest insecticide resistance.⁹

Considerable efforts have been made in conventional breeding and genetically modified programs to breed for resistance against aphids in wheat.^{10–14} Nevertheless, the development of aphid-resistant wheat cultivars has not reached such success to suppress aphid abundance in the fields, as a result of the complexity of plant–aphid–natural enemy interactions.^{10,11,13} Moreover, most wheat cultivars were found to be susceptible to aphid attack, and the resistance genes do not exist in the gene pool of modern wheat varieties.^{15–18} Breeders and growers are still struggling to find an efficient strategy for aphid control by continuously facing the aphid outbreaks in wheat production.^{16,19–21} Then, screening potential genes from wheat germplasm resources for performing crosses between different species, genera, higher-ranking taxa, or by genetic engineering may make it possible to transfer aphid-resistance genes to wheat.

During the long process of suffering from phytophagous insects, plants have evolved a variety of defense mechanisms, including constitutive and inducible defenses, which has cast a new light against aphids.^{22–25} Terpenes, a major class of volatile organic compounds (VOCs), generally play vital roles in repelling herbivores and attracting predators or parasitoid in plant-induced defense.^{26–29}

Volatile terpenoids are mainly synthesized through the mevalonate (MVA) pathway in the cytoplasmic/endoplasmic reticulum or the 2-c-methyl-d-erythritol 4-phosphate (MEP) pathway in the plastids.^{30–32} Geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) as acyclic substrate molecules were converted into mono-, sesqui-, and diterpene skeletons, respectively, by specialized terpene synthases (TPSs).³³ Based on phylogenetics and functional studies, TPSs are commonly divided into eight subfamilies: TPS-a (sesquiterpene synthases), TPS-b (cyclic monoterpene and hemiterpene synthases), TPS-c (copalyl diphosphate synthases), TPS-d (gymnosperm-specific terpene synthases), TPS-e (ent-kaurene synthases), TPS-f (other diterpene synthases), TPS-g (acyclic monoterpenes), and TPS-h (specific to lycopod *Selaginella moellendorffii*).^{34,35} Among these, the number of TPS-g and TPS-b has been *in vitro* identified to catalyze GPP, FPP, and GGPP into monoterpenes, sesquiterpene, and diterpene, respectively.

Linalool is acyclic monoterpene alcohol produced by monoterpene synthase in many plants, which has a sweet floral fragrance.³⁶ The gene synthesizing linalool has been characterized and isolated in many cash crops, including *Camellia sinensis*, *Gossypium hirsutum*, *Citrus unshiu* Marc, *Cinnamomum osmophloeum*, *Vitis vinifera*, and others.^{29,37–40} These results indicated that linalool synthases were able to convert the GPP into S-linalool and FPP into (*E*)-nerolidol.

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Several studies have shown that linalool emission plays important roles in plant defense mechanisms by repelling pests and deterring oviposition. For example, the transgenic *Nicotiana tabacum* L. plants overexpressing the cotton *GhTPS12* gene, which produced a significantly higher level of (3S)-linalool, showed direct defense responses against *Helicoverpa armigera* female adults and *Myzus persicae*.²⁹ Moreover, S-linalool is a key component with defense effects against *Nilaparvata lugens* Stål⁴¹ and is the most abundant VOC emitted from rice plants damaged by the fall armyworm *Spodoptera frugiperda* Smith & Abbot larvae. This cue significantly attracts parasitic wasps.⁴² Hence, linalool may also have potential in the manipulation of plant defense focusing on biological control of herbivores through insect-resistant cultivar breeding.

Thinopyrum intermedium (host) Barkworth & Dewey, as a close relative of wheat, has many valuable attributes such as being multiflorous, resistance to pests and diseases, and tolerance to salt and drought. It is one of the most important wild resources that has been widely used in genetic improvement of wheat.⁴³ The octoploid Trititrigia was selected from the common wheat and *Th. intermedium* (wheat-*Th. intermedium* partial amphiploid) cross. It could comprehend the superior traits of parents and act as a bridge parent for transferring superior genes of *Th. intermedium* grass to wheat.⁴⁴ Based on 2 year field observations on aphid population growth over time and field experiments with the octoploid Trititrigia-resistant level to *S. avenae* during the wheat filling stage, we first hypothesized that some VOCs could be emitted from octoploid Trititrigia and deter *S. avenae* from the host plant. The deterring effects might be enhanced by aphid damages. Then, VOCs were collected and analyzed by gas chromatography associated to mass spectrometry (GC-MS) to compare the chemical profiles and investigate active compounds through olfactory tests. Second, we hypothesized that some terpene synthases might be involved in terpenoid biosynthesis. Therefore, a *de novo* comparative transcriptomic analysis between intact and aphid-damaged octoploid Trititrigia was performed. Third, the biosynthesis process was hypothesized to be mediated by terpene synthase genes. The terpene synthase gene of functional volatile terpenoid was cloned and the enzyme was expressed *in vitro* prior to functional characteristic assessment. These data not only strengthen new insights into plant defense responses to *S. avenae* but also provide a better understanding of the mechanism underlying constitutive and induced defense response of cereal crops to aphids, especially providing applicable candidate resistance genes for breeding wheat cultivars.

MATERIALS AND METHODS

Insects and Plants. The *S. avenae* aphid colony was reared on wheat seedlings (Lumai 21 variety) in a controlled condition chamber (21 °C, 16:8 h photoperiod and 70% relative humidity), with new wheat seedlings provided once a week. Octoploid Trititrigia was provided by Dr. Xingfeng Li from the National Wheat Improvement Center of China. We declared that the collection of the plant materials complies with institutional, national, or international guidelines.

Resistance Assessment and Treatment Design in Fields. Wheat, octoploid Trititrigia, and triticale (intergeneric cross between wheat and rye) were sown in the early October, 2015 and 2016 in fields (35°38'N, 116°02'E) to test their resistance to *S. avenae* according to the Rule for Resistance Evaluation of Wheat to Aphids of China (NY/T 1443.7-2007). Winged and wingless aphids were

recorded on 5 weekly sampling dates over aphid damage periods (from 10 April to 25 May). Common wheat Lumai 21 was considered as control. In 2017, six plots (1 m² each) sowed with octoploid Trititrigia were set at the same location. Plots were individually covered by an insect-proof screen. Three to five plants in each plot were treated by introducing 100 wingless adults of *S. avenae* onto the heads of each plant at the flowering stage with triplicates. The other three plots without aphid infestation served as a control. Both intact and damaged wheat heads were sampled after 72 h.

Volatile Collection and Gas Chromatography-Mass Spectrometer (GC-MS) Analysis. Sampling heads infected by aphids for 72 h were collected in the field. The air entrainment (dynamic headspace collection) was carried out following standard procedures using the flowering heads of octoploid Trititrigia. For each headspace collection, a head was enclosed in a gas sampling bag (polytetrafluoroethylene, 2 L, E-Switch) opened at the bottom, and with two collection ports at both sides (one for the inlet of air and the other for the outlet). The bottom was closed without pressure around the plant stem by a strip. Air was purified by a passage through an activated charcoal filter and was pumped into the vessel through the inlet port at 600 mL·min⁻¹. The air was drawn out at 400 mL·min⁻¹ through 50 mg Porapak Q (Alltech Associates, Carnforth, Lancashire, U.K.) in a 5 mm diameter Tenax glass tube. The difference in the flow rate created a slight positive pressure within the collection bag to prevent unfiltered air from entering into the system. All connections were made with a poly(tetrafluoroethylene) (PTFE) tubing with brass ferrules and fittings were then 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 °C) under a stream of nitrogen for a minimum of 2 h. Volatiles were collected on Porapak Q tubes inserted into the collection port on the top of the vessel and were subsequently eluted with 500 μL of fresh hexane. Air entrainment samples were analyzed by an Agilent 7890A gas chromatograph coupled with a 7000D mass spectrometer (Agilent Technologies, Palo Alto, CA). For air entrainment samples, aliquots of samples (1 μL) were analyzed 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 a carrier gas at 1 mL·min⁻¹; the oven temperature was programmed to increase from 50 to 60 °C (5 min hold) with a rate of 5 °C min⁻¹ and then increased to 250 °C with a rate of 10 °C min⁻¹ (5 min hold). The transfer line temperature was 250 °C and the ion source temperature was 250 °C. Ionization was performed 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 references.

Physiological and Behavioral Assays. The electroantennography (EAG) tests were performed to determine the antennal responses of *S. avenae* to S-linalool as previously described.^{45,46} Antennae were excised from the base and attached immediately with the signal gel (Parker Laboratories, INC) between two electrodes. The base and tip of antennae were connected with the reference electrode and the recording electrode, respectively. For EAG, 10 μL of S-linalool dissolved in *n*-hexane (Kaitong, Tianjin, 98.0%) at the concentrations of 0.01, 0.1, 1, 10, and 100 μg·μL⁻¹ was injected into the filter paper (1 cm × 1 cm) and placed in a Pasteur pipette. Five dilutions of the compound from the lowest to the highest concentrations were tested. Only one concentration was detected for each antenna, and the trials with each EAG preparation were repeated three times. Six antennae were measured for each concentration. *N*-hexane was used as a control before and after each antennae test. The end of the Pasteur sample tube was connected with the stimulus controller (model CS-55, Syntech, The Netherlands). An antenna was placed 1 cm from the end of the airflow tube with a 10 mL·s⁻¹ air rate and stimulus duration of 0.5 s with a 30 s interval. Pressing the foot pedal after baseline balanced was followed by EAG response measurement. The Pasteur pipette and the filter paper were replaced each time when the compound was changed. The relative value of antennal potential response (mV) = Response to odor stimulation (mV) – Average value of response

before and after control test (mV). One-way ANOVA and Tukey's test ($P < 0.05$) were used to analyze whether the EAG reaction values have significant differences.

The behavioral assays were performed using a four-arm olfactometer in a controlled room (21 ± 1 °C and $70 \pm 5\%$ relative humidity). The olfactometer was built in Plexiglas with a 10.5 cm walking area and connected to a vacuum pump to extract air. The flow rate of each arm was $150 \text{ mL}\cdot\text{min}^{-1}$. Volatile chemical was dissolved in *n*-hexane (Kaitong, Tianjin, 98.0%) at five concentrations (0.01, 0.1, 1, 10, and $100 \mu\text{g}\cdot\mu\text{L}^{-1}$) and *n*-hexane was used as a control. One of the four source bottles contained a 1 cm^2 piece of filter paper soaked with $20 \mu\text{L}$ of the tested solution, while the other three bottles contained filter paper soaked with $20 \mu\text{L}$ of *n*-hexane as controls. Each aphid was placed at the center of the exposure chamber and observed for 15 min to record the time spent in each olfactometer area. A total of 20 replicates were performed for each concentration, and each aphid was tested only once. For every two observations, the olfactometer was rotated by 90° to avoid directional bias. Between each treatment, the system was cleaned with pure ethanol and rinsed with distilled water. Test data were expressed as "mean \pm 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.

Transcriptome Analysis of Octoploid Trititrigia Before and After Aphid Infestation. Transcriptome analysis was performed both on intact and aphid-infected wheat heads.⁴⁷ Total RNA was extracted from three biological replicates of the flowering head using a Trizol reagent (Invitrogen) according to the manufacturer's instructions and then treated with RNase-free DNase I (Invitrogen). Each biological replicate contained a single flowering head. The quality and concentration of the total RNA were determined using a NanoPhotometer spectrophotometer (Implen, CA) and the Qubit RNA Assay Kit with a Qubit 2.0 Fluorometer (Life Technologies, CA), respectively. The RNA integrity was confirmed using an Agilent 2100 Bio-Analyzer (Agilent Technologies, CA). RNA samples with $\text{RIN} \geq 7$ and $28\text{S}/18\text{S}$ ratio $\geq 1.5:1$, total amount $\geq 3 \mu\text{g}$, were considered acceptable and used as an 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) according to Illumina manufacturer's instructions. Double-stranded cDNA was generated using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen). 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 200 bp were obtained by deleting low-quality reads with vague nucleotides and filtering adapters from the raw data.

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 a quality value $Q \leq 5$ in a read) were removed. De novo transcriptome assembly was accomplished from all of these clean reads with the Trinity program. Only sequences with no more than two nucleotide mismatches were used for annotation.

To annotate the transcriptome, assembled sequences were further used as query sequences to BLAST with NCBI nonredundant protein sequence (Nr), SwissProt, Kyoto Encyclopedia of Genes and Genomes (KEGG), Eukaryotic Ortholog Groups (KOG), Pfam, Gene Ontology (GO), and NCBI nucleotide sequences (NT) databases, respectively. The best hit of alignment was used to infer the 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 using WEGO software for all of the transcripts. KEGG

pathway annotations were retrieved from the KEGG (<http://www.genome.jp/kegg/>) database.

Analysis of the Differentially Expressed Genes of Octoploid Trititrigia Before and After Aphid Infestation. Using the *de novo* assembled transcriptome data as a reference sequence, the clean reads of each sample were mapped to this reference sequence 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 FPKM method (fragments per kb per million). Using edgeR software, differentially expressed genes (DEGs) were determined between the damaged and intact octoploid Trititrigia head libraries, respectively, and the DEGs were defined as significant based on a false discovery rate (FDR) of ≤ 0.05 and an absolute value of \log_2 ratio ≥ 1 .

The terpene synthase (TPS) genes that were prominently and differentially expressed in the expression profile data were chosen for validation by reverse transcription-quantitative PCR (RT-qPCR) and gene-specific primers were designed by Primer Premier 5. All primer pairs for these qPCR were deposited in additional (Table S1). The qPCR used a Top Green qPCR SuperMix (TransScript) in $20 \mu\text{L}$ reactions according to the manufacturer's instructions. qPCR was performed using the LightCycler96 Real-time PCR System. The qPCR amplification was performed in thermocycler conditions starting with 30 s at 94°C , 5 s at 94°C , 40 cycles of 30 s 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 $2^{-\Delta\Delta\text{Ct}}$ for statistics was applied with three biological replicates.

Cloning and Analysis of the S-Linalool Synthase Gene. The octoploid Trititrigia S-linalool synthase gene (*OtLIS*) sequence was amplified by RT-PCR using the octoploid Trititrigia total RNA as a template. The cDNA was synthesized by a TaKaRa PrimeScript RT-PCR Kit (Dalian, China) and a negative control was set up. The primers were designed according to the transcriptome sequence (Table S1). The full-length ORF of *OtLIS* was cloned by PCR whose amplification was performed using TaKaRa Tks Gflex DNA Polymerase (Dalian, China) in $50 \mu\text{L}$ reactions according to the manufacturer's instructions. The nested PCR amplification was performed in conditions starting with 60 s at 94°C , 30 cycles of 10 s at 98°C , 15 s at 55°C , and 60 s at 68°C . The isoelectric point (pI) and molecular weight (Mw) of *OtLIS* were analyzed by the ExPASy website (http://web.expasy.org/compute_pi/). The protein-coding region of the *OtLIS* 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 neighbor-joining (NJ) tree was conducted using MEGA7.0 and 1000 replicates of bootstrap analyses.

Functional Expression and Enzyme Activity Analysis of *OtLIS*. The ORF of *OtLIS* was connected to the prokaryotic expression vector pet32a (+), and the plasmid was transformed into DH5a cells. The final plasmid was transformed into an *Escherichia coli* dialytic cell-free protein expression system (TSINGKE), inoculated at 25°C for 16 h, and the protein was expressed according to the instructions. The supernatant was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Enzyme activity analysis was performed according to the procedure described by Huang et al.²⁹ GPP, FPP, and GGPP were used as substrates for enzyme assays. The enzyme activity products were analyzed by GC-MS.

RESULTS

Resistance Assessment, Volatile Collection, and Identification of Octoploid Trititrigia. According to the 2 year field observation on aphid population growth over time, significant decreases of alate and apterous *S. avenae* abundances were observed in octoploid Trititrigia plants compared to the triticales and the control (Figure 1), which

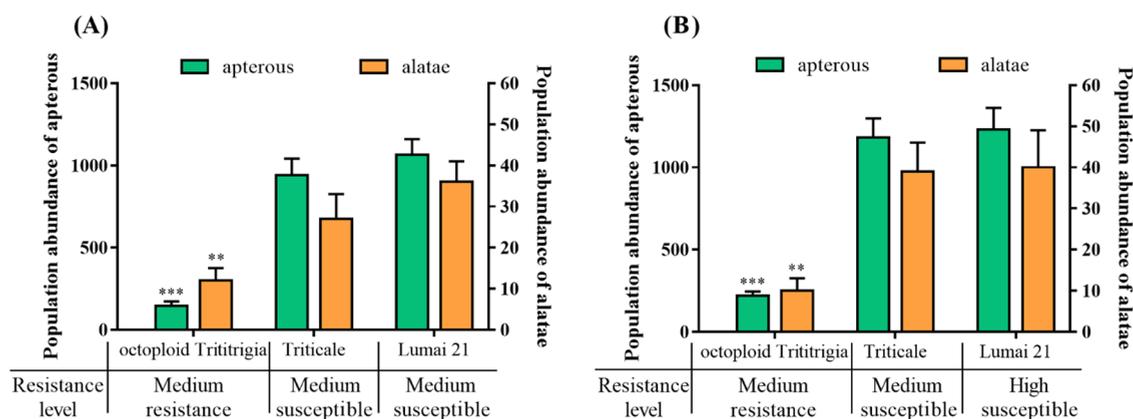


Figure 1. Field tests of the octoploid Trititrigia resistance level to *Sitobion avenae* both in 2016 (A) and in 2017 (B). The abundances of apterous aphids and alatae were analyzed separately. The left Y-axis represents the abundance of the apterous and the right Y-axis represents the abundance of alatae. Values are mean \pm standard error. **, $P < 0.01$ and ***, $P < 0.001$.

revealed that *S. avenae* might have nonpreference performance with medium resistance to octoploid Trititrigia during the wheat filling stage (Figure 1). GC-MS analysis showed that the release of seven volatile compounds changed after *S. avenae* infested octoploid Trititrigia, including S-linalool, δ -cadinene, (E)-nerolidol, ent-kaurene, β -Myrcene, (E)-3,8-dimethyl-1,4,7-nonatriene (DMNT), and β -caryophyllene. Among them, the amount of S-linalool increased significantly after aphid infestation (Figure 2). So, we focused on linalool and analyzed its resistance properties thereafter.

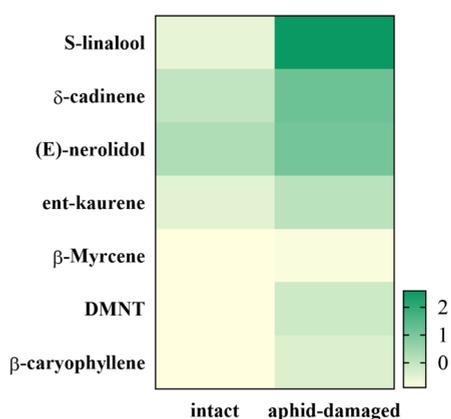


Figure 2. Peak areas of volatile organic compounds (VOCs) emitted from intact and aphid-damaged wheat heads. The value was standardized by the Z-score.

Response of *S. avenae* to S-Linalool. The EAG response of *S. avenae* to S-linalool had significant differences among the five concentrations ($F = 4.742$, $P = 0.006$), and the highest EAG response was obtained when the concentration was $1 \mu\text{g}\cdot\mu\text{L}^{-1}$ (Figure 3A). Olfactory response tests showed that $1 \mu\text{g}\cdot\mu\text{L}^{-1}$ ($W = -3.385$, $P < 0.05$) and $10 \mu\text{g}\cdot\mu\text{L}^{-1}$ ($W = -3.352$, $P < 0.01$) of S-linalool had a significant repellent effect on *S. avenae* (Figure 3B).

Strategy for De Novo Assembly and Transcriptomic Data Analysis. We used the samples of the octoploid Trititrigia head and produced 42.6 Gb of clean data. We obtained 243718 unigenes after sequence assembly. The total length of the transcribed sequence was 203088986 bp, the average length was 833 bp, and the N50 size was 1041 bp with

47.09% GC content. The assembled transcript sequence lengths ranged from 300 to 3000 bp, and the dominant length of the sequences was 300–400 bp (Figure S1). In addition, the number of transcripts of sequence length ≥ 3000 bp was 3863.

For a comprehensive understanding of the role of the interesting gene, the detected genes were functionally annotated in the databases of NR public database, Swissport, GO, KEGG, KOG, and NT. A total of 182 348 (74.8%) unigenes could be annotated into at least one of the six databases. Among them, 85 145 (34.94%), 73 806 (40.48%), 53 526 (21.96%), 125 347 (51.47%), 138 277 (56.74%), and 68 267 (28.01%) unigenes were BLAST to GO, KEGG, KOG, NR, NT, and Swissport, respectively.

Based on the FPKM method, the transcript abundance of each gene from the intact octoploid Trititrigia heads and the aphid-damaged heads was analyzed. A total of 233023 DEGs were identified between the intact and damaged octoploid Trititrigia head libraries. These DEGs were assigned to GO (gene ontology) classes to explore their functions (Figure S2). Out of these DEGs, significantly upregulated genes in octoploid Trititrigia heads induced by *S. avenae* were identified. Candidate resistance genes were identified from overlapping TPS genes; TPS functions as a central regulator in resistance against *S. avenae* (Figure 4).

Candidate Genes Related to S-Linalool Biosynthesis.

The levels of transcripts encoding the S-linalool synthase (LIS) of aphid-damaged octoploid Trititrigia heads were significantly higher than those of intact octoploid Trititrigia heads. These were further validated using RT-qPCR. The levels of genes encoding LIS were significantly higher in the aphid-damaged octoploid Trititrigia than those of intact octoploid Trititrigia heads (Figure 5). Based on RT-qPCR results, the expression profiles of the seven genes were identified by the Illumina sequencing or RT-qPCR analysis, but the accurate folding change of the two methods was biased. This suggested relative rationality and accuracy of the transcriptome analysis in the present study. The expression of TR127690_c3_g3 was most significantly increased after aphid infection and was selected for further study.

Characterization and Phylogenetic Analysis of *OtLIS*.

GC-MS analysis showed that S-linalool was the only volatile compound with a significant difference in the amount emitted from the aphid-damaged octoploid Trititrigia heads. We cloned the complete ORF of *OtLIS* by nested PCR. The

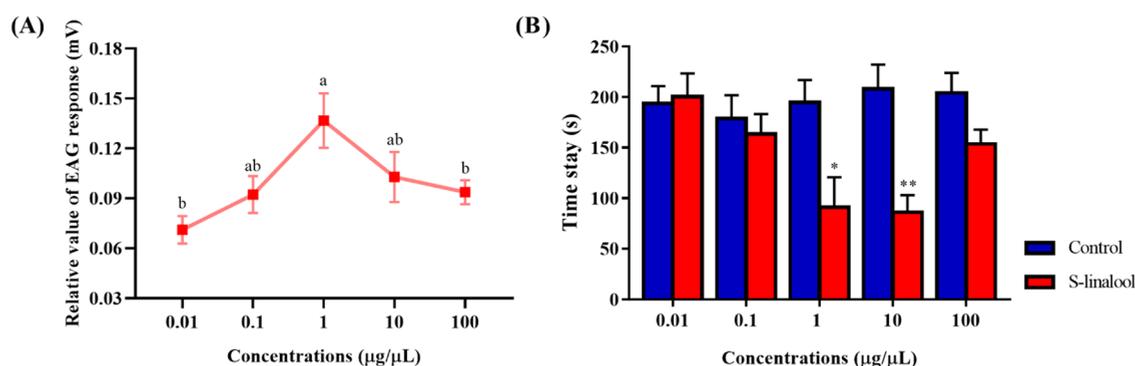


Figure 3. Responses of *Sitobion avenae* to S-linalool. (A) Electroantennography responses (mean \pm SE) of *S. avenae* to S-linalool. Error bars represent the standard errors of six replicates. Lowercase letters indicate the significant difference ($P < 0.05$). (B) Olfactory responses of the *S. avenae* to S-linalool. * and ** indicate the significant difference ($P < 0.05$) and the extremely significant difference ($P < 0.01$) between treatment and control.

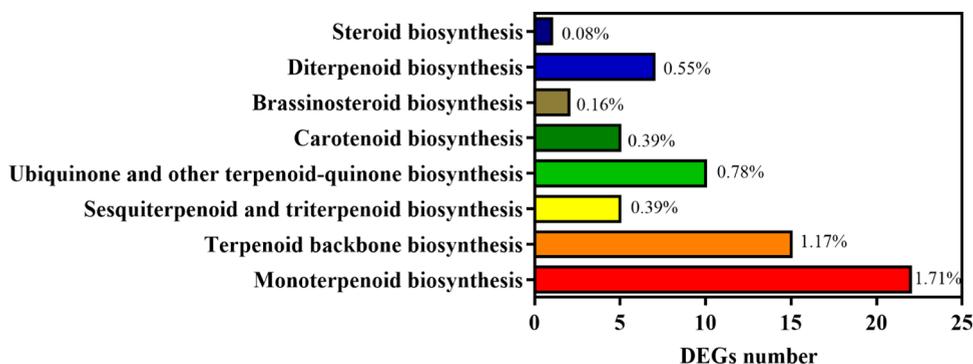


Figure 4. Analysis of differentially expressed genes involved in terpenoid anabolism in the KEGG pathway between intact octoploid *Trititrigia* heads and aphid-damaged heads.

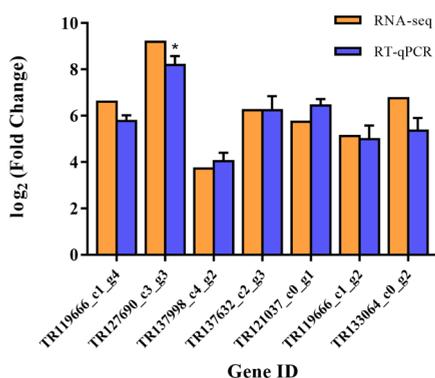


Figure 5. Expression level of S-linalool biosynthesis genes using the RT-qPCR, in comparison to corresponding data detected in the RNA-Seq. The relative expression ratio of each DEG is presented in a log₂ value of the aphid-damaged head vs the intact octoploid *Trititrigia* head libraries. The values for mean \pm SE ($n = 3$). Data were normalized to the *TaActin* gene and vertical bars indicate standard deviation. * indicates the significant difference ($P < 0.05$) between the relative expression of genes.

complete ORF of *OtLIS* was 1680 bp, within a predicted Mw of 64.19 kDa, coding 559 amino acids (aa), and had a pI of 5.9. The amino acid sequence alignment results show that *OtLIS* contained an aspartate-rich region DDxxD motif (Figure 6). The sequence data have been submitted to the GenBank database under accession number MZ388428.

OtLIS clustered with other angiosperm monoterpene synthases (TPS-g family) based on the phylogenetic relation-

ship analysis (Figure 7). There was no difference in the phylogenetic relationship of the *OtLIS* family from that of the current plant. Furthermore, the LIS 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.

Functional Validation of *OtLIS*. The function of *OtLIS* was analyzed according to the potential substrate (GPP, FPP, GGPP); GC-MS results showed that recombinant protein *OtLIS* could catalyze the formation of S-linalool from GPP and (*E*)-nerolidol from FPP in vitro (Figure 8). No enzyme activity was detected in the reaction system with GGPP substrates.

DISCUSSION

Breeding insect pest-resistant varieties is an efficient pest-controlling strategy for agricultural crops. Screening insect pest-resistant germplasm and mining-resistant genes are the basis of breeding-resistant varieties. Wheat is the major crop worldwide that is severely damaged by aphids, which leads to yield and quality reduction, while the conventional varieties of wheat lack aphid-resistant genes. The medium resistance of octoploid *Trititrigia* to *S. avenae* was determined since the numbers of *S. avenae* including the apterous aphids and alatae on the octoploid *Trititrigia* plant were far less than those of others, and the resistance mechanism was likely nonpreferable (Figure 1). It indicated that the octoploid *Trititrigia* plant may produce volatile chemical compounds that had a repellent effect on *S. avenae* in the wheat canopy. Our study showed that S-linalool was emitted from both intact octoploid *Trititrigia*

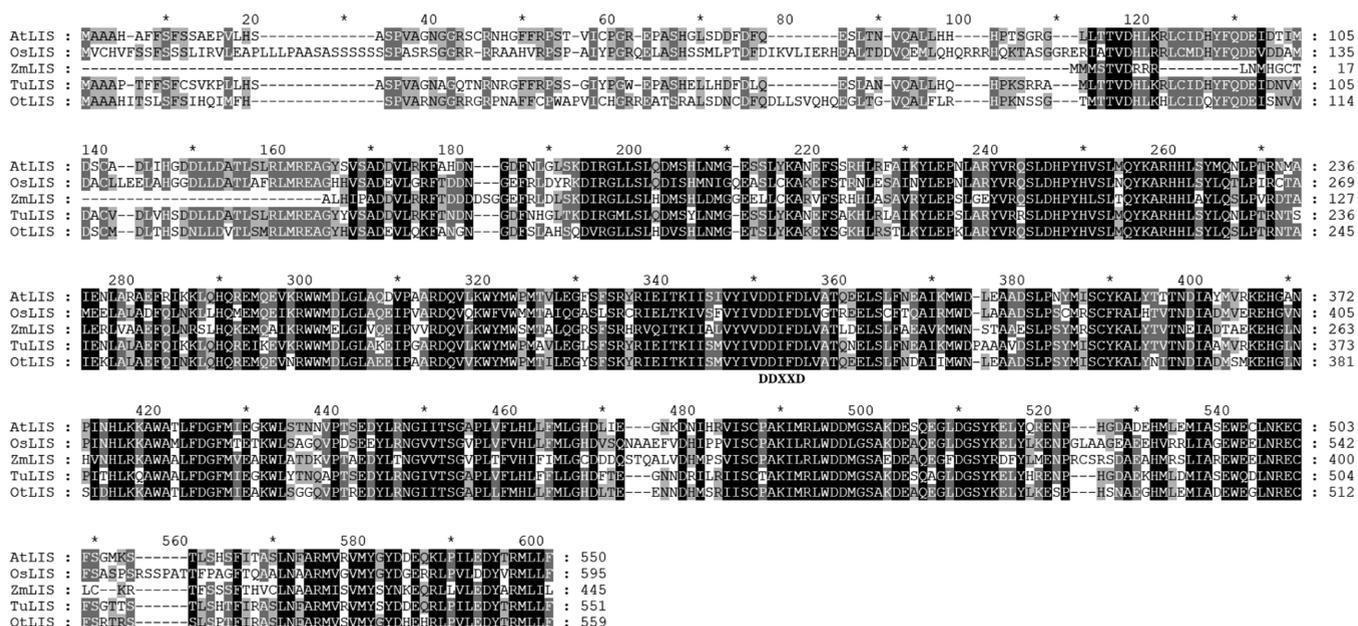


Figure 6. Alignment of OtLIS, *Oryza sativa* LIS (XP_015623808), *Zea mays* LIS (AQK60700), *Triticum Urartu* NES1 (EMS50987), and *Arabidopsis thaliana* LIS (NP_176361) amino acid sequences. The highly conserved regions are DDxxD. The alignment was performed by CLUSTALW.

Tree scale: 0.1

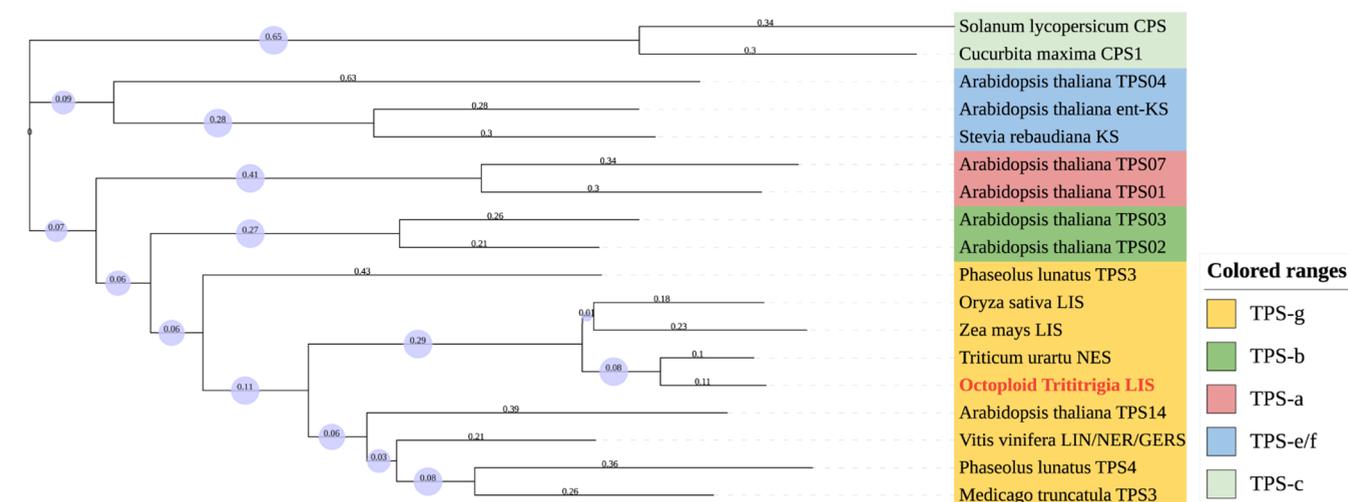


Figure 7. Phylogenetic tree of OtLIS together with known TPSs from other plants. OtLIS fell in the cluster of angiosperm monoterpene synthases (TPS-g). The functional descriptions and accession numbers of these genes are listed in Table S2.

heads and *S. avenae*-damaged heads from which a higher amount was emitted. It revealed that the *S. avenae* feeding could induce the biosynthesis of the volatile terpenoid to produce a large amount of S-linalool, which enhanced the defensive effect on *S. avenae*.

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 defense against herbivorous insect pests.^{29,42} Genetical engineering *Arabidopsis thaliana* plants released S-linalool that repelled *M. persicae*.⁴⁸ 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 brown planthopper *N. lugens*.⁴¹ In this study, we demonstrated that S-linalool (1 and 10 $\mu\text{g}\cdot\mu\text{L}^{-1}$) had a

remarkable repellent effect on *S. avenae* by the four-arm olfactory test. This could provide a new semiochemical for exogenous application with aphid avoidance in the field.

Some TPSs have been identified, and even successfully used to increase direct plant defense^{29,49} or indirect plant defense by attracting natural enemies of the herbivores.^{50–52} A few TPSs have been discovered in the field crops,^{13,53,54} which have greatly promoted the understanding of the mediation of terpene compound biosynthesis. Meanwhile, studies have reported that the expression levels of TPSs were significantly correlated with pest-defense mechanisms in the plants.^{29,55,56} Then, we used transcriptomic data of the intact and aphid-damaged octoploid *Triticum* heads to screen the regulatory genes in the terpene biosynthetic pathway.⁴⁷ This assembled transcriptome could be used as a database of the wheat

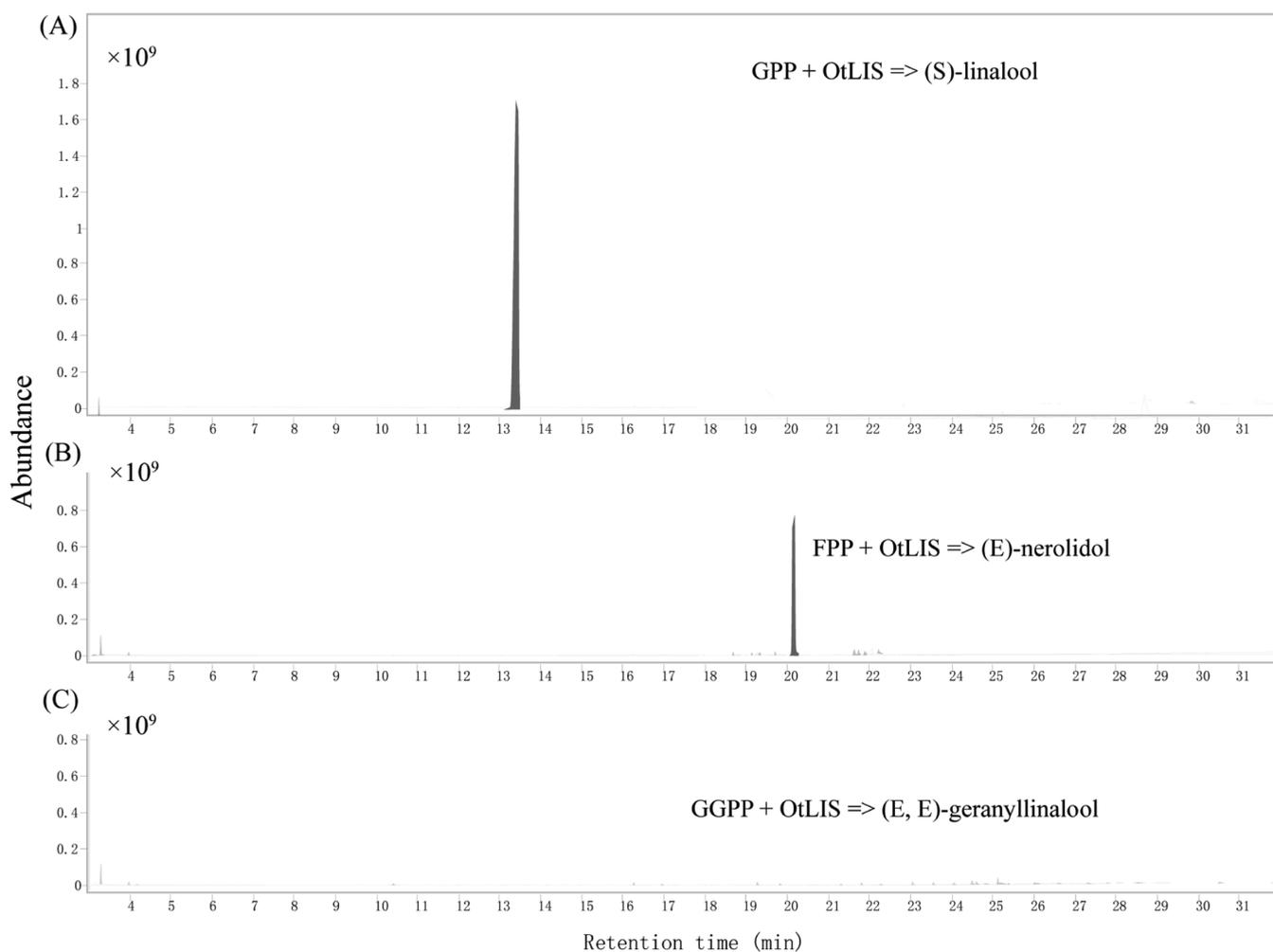


Figure 8. GC-MS analysis of the volatile products of recombinant OtLIS. The protein was heterologously expressed in *E. coli*. The expression products of OtLIS after the incubation with GPP (A), FPP (B), and GGPP (C).

germplasm to search for high-quality aphid-resistant genes and provide more molecular resources for future functional characterization analysis of aphid resistance.

The emission of the volatile terpenoid S-linalool was higher in aphid-fed octoploid *Trititrigia* heads than untreated heads. At the same time, the transcripts of S-linalool synthase genes were also the most abundant. However, little has been known about the molecular mechanisms controlling S-linalool biosynthesis in wheat. In this study, the *OtLIS* sequence was obtained, and amino acid sequence alignment of *OtLIS*, *Oryza sativa* LIS (XP_015623808), *Zea mays* LIS (AQK60700), *Triticum urartu* LIS (EMS50987), and *A. thaliana* LIS (NP_176361) revealed the typical conserved motifs and domains of the specific terpene synthase (Figure 6). *OtLIS* contained an aspartate-rich region DDxxD motif, which was described as a function involved in substrate binding to coordinate divalent metal ions.⁵⁷ Phylogenetic analysis demonstrated that the *OtLIS* and *TuNES1* were highly homologous. The results could help to provide a deeper understanding of how changes in the gene expression are related to the volatile terpenoid S-linalool changes in the octoploid *Trititrigia* heads.

Plants have evolved a series of defense mechanisms against biotic stressors.⁵⁸ In addition to terpenes, plants release a spectrum of VOCs including fatty acids, phenylpropanoids,

green leaf volatiles, and glucosinolate breakdown products.^{59–61} At present, several pest management strategies have been developed based on VOC-mediated plant–insect interactions, such as push–pull systems, synthetic odorant traps, and crop cultivars with modified VOC profiles.⁶² Our study is of great significance, showing that *OtLIS*, which encodes terpene synthase, confers resistance to a wheat aphid by S-linalool emission. The deployment of *OtLIS* for breeding aphid-resistant wheat cultivars could alleviate both the yield loss and the virus transmission problems for growers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c05978>.

Primers used in the quantitative real-time PCR and nested PCR and the complete ORF of octoploid *Trititrigia* S-linalool synthase gene, protein sequences used for phylogenetic analysis of the plant TPSs, length distribution of the assembled transcript sequences of octoploid *Trititrigia* heads, and the number of DEGs associated with biological processes in Gene Ontology (GO) classification (PDF)

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Notes

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ABBREVIATIONS USED

DMNT, (E)-3,8-dimethyl-1,4,7-nonatriene; EAG, electroantennography; FPKM, fragments per kb per million; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; LIS, S-linalool synthase; MEP, 2-c-methyl-d-erythritol 4-phosphate; MVA, mevalonate; Mw, molecular weight; OtLIS, octoploid *Trititrigia* S-linalool synthase; pI, isoelectric point; PTFE, poly(tetrafluoroethylene); TPS-a, sesquiterpene synthases; TPS-b, cyclic monoterpene and hemiterpene synthases; TPS-c, copalyl diphosphate synthases; TPS-d, gymnosperm-specific terpene synthases; TPS-e, ent-kaurene synthases; TPS-f, other diterpene synthases; TPS-g, acyclic monoterpenes; TPS-h, specific to lycopod *Selaginella moellendorffii*; TPSs, terpene synthases; VOCs, volatile organic compounds

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