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# Induced Bowman-Birk trypsin inhibitor TaBBTI-1 in wheat during poor-host interactions with aphids confers broad-spectrum resistance to insect herbivores

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ABSTRACT

Aphids are phloem-feeding insects that cause significant yield losses on major crops such as wheat. While non-host resistance is considered the most durable and efficient immune system of plants, its mechanisms against aphids remain poorly understood. Here, aphid bioassays and electrical penetration graphs allowed to classify *Sitobion avenae* as a host, *Myzus persicae* as a poor-host, and *Acyrthosiphon pisum* as a non-host on wheat. Pre-infestation by *M. persicae* induced stronger wheat resistance during poor-host interactions, significantly reducing subsequent aphid nymph production compared to *S. avenae* pre-infestation. Comparative transcriptomic analysis of wheat under host versus poor-host interactions uncovered that the upregulated genes induced by *M. persicae* infestation primarily associated with alpha-linolenic acid metabolism and jasmonic acid (JA) biosynthesis pathways. Phytohormone quantification showed that *M. persicae* feeding, but not *S. avenae*, elevated endogenous levels of JA in wheat. Notably, a Bowman-Birk trypsin inhibitor of wheat, designated as *TaBBTI-1*, was specifically and strongly induced by *M. persicae*, and exogenous JA application to wheat plants dramatically upregulated *TaBBTI-1*. Feeding on artificial diets supplemented with recombinant TaBBTI-1 protein and wheat leaves overexpressing *TaBBTI-1* significantly impaired the fecundity of *S. avenae*. Furthermore, ectopic expression of *TaBBTI-1* in *Nicotiana tabacum* dramatically reduced host susceptibility to *M. persicae* and lepidopteran pest *Helicoverpa armigera*, demonstrating broad-spectrum resistance against herbivorous insects. This study provides novel insights into the molecular mechanisms underlying non−/poor-host resistance of wheat against aphids and development of novel and durable pest control in crop plants.

## 1. Introduction

Aphids are among the most destructive agricultural pests globally, causing yield losses in a wide range of crops through direct sap-feeding and transmission of plant viruses [1]. Chemical pesticides remain the dominant method for aphid control, however, their overuse accelerates the evolution of insecticide resistance and causes environmental contamination, highlighting an urgent need for sustainable alternatives [2]. The utilization of host-plant resistance or plant defenses against herbivory represents an economically and ecologically sustainable pest management strategy.

Insect herbivory attacks activate defensive pathways mediated by phytohormones such as jasmonic acid (JA), salicylic acid (SA), ethylene, and abscisic acid along with signaling molecules including reactive oxygen species (ROS) in plants [3]. This induction stimulates the biosynthesis and accumulation of defensive secondary metabolites, such as benzoxazinoids and protease inhibitors (PIs), ultimately impairing insect growth and development [4]. Hemipterans, such as aphids, have highly modified piercing sucking mouthparts that can penetrate extracellular components and eventually feed on phloem sap from sieve elements. In compatible plant insect interactions, most research has demonstrated that feeding by hemipterans (e.g., aphids) induces SA-dependent defense responses, which parallel plant responses to microbial pathogen infection [5]. However, some studies have also

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demonstrated that feeding by the greenbug *Schizaphis graminum* on wheat *Triticum aestivum* or *sorghum Sorghum bicolor* concurrently upregulates key genes and elevates hormone levels in both SA- and JA-signaling pathways [6,7]. Additionally, many resistance loci/genes to aphids have been cloned in host plants, but their durability is limited due to insect adaptation [8]. *Mi-1.2*, an SA-associated single dominant gene present in tomato cultivars, confers resistance against certain phloem-feeding herbivores including the tomato aphid *Macrosiphum euphorbiae*, whitefly *Bemisia tabaci*, psyllid *Bactericera cockerelli* and root-knot nematodes [9–11], but was observed to lose resistance at high soil temperatures [12]. Recently, above 15 genes conferring resistance to the Russian wheat aphid *Diuraphis noxia* have been characterized; however, resistance in widely commercialized cultivars mediated by the *Dn4* gene has been overcome following an emergence of the new virulent aphid biotype, RWA2 [8].

Non-host resistance represents the most prevalent and durable plant defense strategy against pathogens [13,14]. Non-host-implicated genes have been successfully applied in plant breeding against pathogens. For instance, transgenic rice expressing the maize *Rxo1* gene exhibited strong resistance against *Xanthomonas oryzae* pv. *oryzicola* [15], and transgenic tomato expressing the pepper *Bs2* gene achieved stable resistance against multiple strains of *X. campestris* pv. *vesicatoria* [16]. Most aphid species exhibit narrow host specificity confined to a few plant families [1]. Analogous to the infection phase of pathogens on non-host plants, aphids also attempt to penetrate the leaf surface using stylets regardless of plant species, implying that resistance induction in non-host plants occurs at the molecular level [3,17]. The bird cherry-oat aphid *Rhopalosiphum padi* is unable to survive on *Arabidopsis*, but stylet penetration activity on the leaf surface still occurs during *R. padi-Arabidopsis* non-host interactions [18]. *Arabidopsis* transcriptome analyses identified differentially expressed genes (DEGs) during host versus *R. padi-Arabidopsis* non-host interactions, including vegetative storage protein 1 (VSP1), which contributes to non-host resistance against aphids [18]. Several aphid species, such as the green peach aphid *Myzus persicae*, are highly polyphagous. This species can feed on >400 host plant species, including several major crop types [1,19]. Although there is no documented evidence confirming that *M. persicae* infests cereal crops in the field, it can feed on wheat, barley, and rye with low levels of colonization and limited phloem sap ingestion under greenhouse conditions [20]. Notably, plant thionin genes are strongly upregulated in *M. persicae*-barley poor-host interactions, conferring aphid resistance [21].

Bread wheat *T. aestivum* is a globally significant crop, and wheat aphids severely threaten wheat production worldwide, with the grain aphid *Sitobion avenae* being the most dominant species in wheat fields in China [22–24]. During compatible interactions between *S. avenae* and its natural host wheat, plants initiate varied defense responses to aphid feeding, including SA signaling pathway activation, callose deposition, ROS bursts, and secondary metabolite accumulation [3,25]. However, few studies to date have addressed the mechanisms underlying wheat resistance against non-adapted insect herbivores. In the present study, we demonstrate that infestation by *M. persicae* specifically activates the JA-mediated defense pathway and induces a Bowman-Birk trypsin inhibitor, TaBBTI-1 in wheat during poor-host interactions. Interestingly, this induced TaBBTI-1 confers broad-spectrum resistance against aphids and lepidopteran pests, offering novel insights into aphid-plant interactions and candidate insect-resistance genes for crop breeding.

## 2. Materials and methods

### 2.1. Insects and plants

Clones of *S. avenae* and *A. pisum* were established from a single aphid collected from a field in Langfang city, Hebei province, Northern China, and clones of *M. persicae* were established from a single aphid collected from a tobacco field in Kunming city, Yunnan province, Southwestern China. The aphid populations used for this study were respectively maintained on host plants, including wheat *T. aestivum* L. (var. Zhongmai175), broad bean *Vicia faba* (var. Lincan204) and tobacco *Nicotiana benthamiana* and *N. tabacum* (var. Yunyan) under laboratory conditions (L: D = 16 h: 8 h; 20 ± 1 °C). Cotton bollworms *H. armigera* larvae were collected from a field in Xinxiang, Henan Province, China, and maintained in an incubator under laboratory conditions (L: D = 14 h: 10 h; 25 ± 2 °C; relative humidity: 60% ± 10%) on artificial diet [26]. The seedlings of wheat (12-day-old), broad bean (12-day-old) and tobacco (28-day-old) were used to examine the adaptability of aphids to different host plants under controlled conditions (L: D = 16 h: 8 h; 20 ± 1 °C).

### 2.2. Aphid choice assays

Host preference of *S. avenae*, *M. persicae* and *A. pisum* on different host plants were examined by using aphid choice assays as described previously [27]. Briefly, the leaves of wheat, broad bean, and tobacco were cut into leaf segments with the same size (length 3 cm; width 0.5 cm), the bases of the segments were wrapped with wet cotton to keep fresh. All leaves were then placed in a petri dish with a diameter of 20 cm at equal distances. Forty apterous adults of *S. avenae*, *M. persicae* and *A. pisum* were respectively placed in the center of the petri dish, and the number of aphids settled on each host plant at 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, and 6 h were recorded. Choice assays were carried out in environmental chamber under controlled conditions (L: D = 16 h: 8 h; 20 ± 1 °C). Ten biological replicates were performed for each group. The differences among groups were examined using one-way analysis of variance (ANOVA) test, followed by Duncan's multiple comparisons.

### 2.3. Detection of callose deposition and $H_2O_2$ accumulation in wheat leaves

At the two-leaf stage, wheat plants were infested with thirty adult aphids of the species *S. avenae*, *M. persicae* and *A. pisum*, and leaves were collected at 6 h and 24 h post-infestation, respectively. Callose deposition in wheat leaves was visualized using aniline blue staining according to protocols reported previously [28]. The stained leaves were photographed with an Echo Revolve Hybrid Microscope (Echo Laboratories, San Diego, CA). Hydrogen peroxide ($H_2O_2$) accumulation was examined in wheat leaves by 3′-diaminobenzidine (DAB) staining according to the histochemical methods described by [29]. $H_2O_2$ accumulation was observed using an Olympus SZX-16 (Olympus Corporation, Tokyo, Japan). Three replicates were conducted for each treatment.

### 2.4. Aphid performance on different host plants

Aphid-susceptible wheat (var. Zhongmia175) at two-leaf stage were infested with two second-instar aphids of *S. avenae*, *M. persicae* and *A. pisum* respectively. The total number of aphids produced was recorded at 8 days and 14 days post-infestation to examine the adaptability of different aphid species on wheat plants. To further compare the colonization of *M. persicae* on wheat and tobacco, 4-week-old tobacco plants and 12-day-old wheat plants were challenged with second-instar aphids, and the total number of aphids was recorded at 8 days and 14 days of feeding. The weights of adult *M. persicae* reared on tobacco and wheat plants for 14 days were also measured. To compare the feeding amounts of *S. avenae* and *M. persicae* on wheat plants, the weight of honeydew excreted by these two species after 48 h of feeding was measured. Briefly, a 20% agarose solution was heated and poured into 9 cm diameter Petri dishes. After cooling, wheat leaves were fixed onto the agarose surface. Thirty adult aphids were placed in each Petri dish to feed on the wheat leaves, with Parafilm positioned beneath each dish to collect honeydew. The weight of the Parafilm was measured before and after aphid infestation to calculate honeydew secretion. Thirty biological replicates were performed for each treatment. To compare the effects of feeding on wheat pre-infested with *S. avenae* versus *M. persicae*

on aphid performance, wheat plants (two-leaf stage) were infested with thirty adult *S. avenae* and *M. persicae* adults for 48 h firstly. *S. avenae*-pre-infested and *M. persicae*-pre-infested wheat were challenged with one *S. avenae* adult. The total number of aphids produced was recorded at 5 days post-infestation. Twenty biological replicates were performed for each treatment. The differences between two groups were examined using Student's *t*-test. All experiments were performed in environmental chamber under controlled conditions (L: D = 16 h: 8 h; 20 ± 1 °C).

### 2.5. Monitoring of aphid feeding behavior using electrical penetration graph (EPG)

The probing and feeding activities of *S. avenae*, *M. persicae*, and *A. pisum* on wheat were monitored using a Giga-8 DC-EPG system (EPG Systems, Wageningen, Netherlands) within a grounded Faraday cage (60 cm × 60 cm × 70 cm). Prior to recording, apterous adult aphids were starved for 1 h in Petri dishes with moist filter paper to standardize feeding motivation. Each aphid was carefully connected to a 3 μm gold wire electrode (20 mm length) using water-soluble silver conductive paint, while a second electrode was inserted into the soil near the wheat root system. Continuous 8 h recordings were conducted under controlled conditions: photoperiod 16 L: 8 D, temperature 20 ± 1 °C, relative humidity 65 ± 5%. Raw signals were processed and annotated using Stylet+a software (version 2.3, EPG Systems) according to established waveform classifications as described previously [30]: np: non-probing phase; C: stylet pathway activity; pd.: potential drop (indicating cell puncture); E1: Salivation into phloem sieve elements; E2: Sustained phloem sap ingestion; G: xylem sap consumption. Individual aphid-plant pairs were used only once. Fifteen independent biological replicates were performed for each treatment. EPG data were analyzed by a Mann-Whitney *U* test.

### 2.6. Whole-transcriptome RNA sequencing of wheat in response to Sitobion avenae and Myzus persicae infestation

At the two-leaf stage (12-day-old), thirty apterous either *S. avenae* or *M. persicae* adults were placed into clip cages (2.7 cm × 2.7 cm × 2.7 cm) on the second leaves of wheat plants for feeding. Aphids were gently removed from wheat leaves using a soft brush at 6 h and 24 h post aphid infestation. Aphid infested leaf tissues were cut off with sterilized scissors, transferred to a 1.5 mL centrifuge tube after surface sterilization, and placed in liquid nitrogen for total RNA extraction. Total RNA of wheat leaves infested with *S. avenae* and *M. persicae* was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). The sample purity was evaluated using NanoPhotometer spectrophotometer (Thermo Fisher, USA), and RNA integrity was verified through RNA Nano 6000 assay kit (Agilent Technologies, CA, USA). The mRNA enrichment was accomplished using Oligo (dT) magnetic beads, after which isolated mRNA was randomly fragmented with divalent cations by fragmentation buffer to prepare a cDNA library for Illumina sequencing. Clean data was obtained from raw data through data filtering, sequencing error rate detection and GC content detection. The clean reads were mapped to a wheat reference genome (version 1.0) (http://plants.ensembl.org/Triticum_aestivum/Info/Index/) using HISAT2. DESeq2 was used to analyze the differential expression between the sample groups and *P*-values were adjusted by Benjamini-Hochberg method to get False Discovery Rates (FDR). Thresholds for significant differentially expressed genes (DEGs) were set at adjusted *P*-values of ≤0.05 and |Log2 (fold change) | values of ≥1. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed for Cluster Profiler package in R version 3.8.1. A total of five treatments were established, including *S. avenae* infestation for 6 h (Sa6h), *S. avenae* infestation for 24 h (Sa24h), *M. persicae* infestation for 6 h (Mp6h), *M. persicae* infestation for 24 h (Mp24h), and uninfested control groups (0 h). Three independent biological replicates were conducted for each treatment.

### 2.7. Measurement of phytohormone contents in wheat infested with Sitobion avenae or Myzus persicae

JA and SA contents in aphid-infested wheat leaves were examined as described previously [31]. Briefly, approximately 0.2 g of wheat leaves infested with thirty apterous *S. avenae* and *M. persicae* adults were harvested after 6 h and 24 h of aphid feeding. Phytohormone extraction was performed by adding 10 times the sample volume of acetonitrile solution, followed by overnight extraction and centrifugation at 12000*g* for 5 min at 4 °C. The supernatants were passed through C18 solid phase extraction column and centrifuged at 10000*g* for 5 min. The supernatants were evaporated and the final extracts were dissolved in 200 μL methanol. The supernatants were collected and used for further detection by a liquid chromatography-tandem mass spectrometry system (LC-MS/MS). Wheat leaves without aphid infestation were set as control groups. A total of three replicates were performed for each treatment. The differences among groups were examined using one-way ANOVA followed by Duncan's test.

### 2.8. Exogenous application of jasmonic acid to wheat plants

JA was initially dissolved in ethanol to 1 M and subsequently diluted with distilled water to prepare aqueous solutions at final concentrations of 0.2 mM, 0.5 mM, and 1.0 mM. Each wheat plant was sprayed with 1 mL of the prepared JA solution using a handheld sprayer. Plants sprayed with aqueous solutions without JA served as the negative control. Leaf samples were collected from treated plants at two time points: 6 h and 24 h post-application. Immediately following collection, the leaf tissues were frozen in liquid nitrogen and stored at −80 °C until used for total RNA extraction. Each treatment included three independent biological replicates.

### 2.9. RT-qPCR analysis

To validate the transcriptome sequencing results, the expression levels of six defense-related DEGs including *Bowman-Birk trypsin inhibitor* (*TaBBTI-1*), *phenylalanine ammonia-lyase* (*TaPAL*), *lipoxygenase* (*TaLOX*), *allene oxide synthase* (*TaAOS*), and *trimethyltridecatetrane synthase* (*TaTMTT*) in wheat plants infested with *M. persicae* and *S. avenae* using RT-qPCR. Additionally, the expression profiles of JA-associated defense genes *TaLOX*, *TaAOS*, *fatty acid desaturase* (*TaFAD*) and *TaBBTI-1* in wheat plants were examined following exogenous JA treatment.

Total RNA was isolated from wheat leaf tissues using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using the HiScript III First-Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech, China). RT-qPCR amplification was performed in 20 μL reaction volumes on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) under the following thermal cycling conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, as previously described [32]. All primer pairs (Table S1) were designed with Beacon Designer 7 software to generate 100–150 bp amplicons. Each experimental group included three biological replicates, with three technical replicates per sample. Data were analyzed by one-way ANOVA followed by Duncan's test. Relative gene expression was normalized to the *TaActin* reference gene and calculated using the $2^{-\Delta\Delta CT}$ method [33].

### 2.10. Sequence analysis and 3D structural modeling

The signal peptide of TaBBTI-1 was identified by using SignalP 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). The prediction of transmembrane regions was performed using TMHMM 2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/). Phylogenetic analysis of TaBBTI-1 and its homologous amino acid sequences was constructed by the neighbor-joining method via MEGA7.0 software.

Bootstrap values were calculated as a percentage from over 1000 replications. The amino acid sequences of all BBTI were listed in Table S2. The TaBBTI-1 amino acid sequence was uploaded to the SWISS-MODEL homology modeling online server (http://swissmodel.ExPASy.org) for 3D structures construction. The three-dimensional model of TaBBTI-1 was predicted by AlphaFold model (M0Y075) with a GMQE value of 0.87 and an identity of 86.75%.

### 2.11. Subcellular localization of TaBBTI-1 in Nicotiana benthamiana

The full coding region of *TaBBTI-1* without the signal peptide (*TaBBTI-1*$^{-sp}$) were cloned into the expression vector pCAMBIA1300. *Agrobacterium* GV3101 carrying pCAMBIA1300: *TaBBTI-1*$^{-sp}$*-GFP* and pCAMBIA1300: *GFP* constructs were resuspended in infiltration buffer [100 μM acetosyringone, 10 mM 2-(N-morpholino) ethanesulfonic acid and 10 mM $MgCl_2$] to an OD600 of 0.6. *Agrobacterium* carrying the constructs were then infiltrated into the leaves of 4-week-old *N. benthamiana* plants using a 1 mL syringe. The infiltrated plants were grown and maintained in growth chambers under controlled conditions (L: D = 16 h: 8 h; 24 ± 1 °C). GFP signal was observed using a Zeiss LSM 880 laser confocal microscope (Zeiss, Jena, Germany) at 488 nm at 36 h post-infiltration. Transient overexpression of *TaBBTI-1*$^{-sp}$*-GFP* or *GFP* in *N. benthamiana* was detected by western blot using anti-GFP polyclonal antibody (ABclonal, China). Total protein of *N. benthamiana* leaves inoculated with *Agrobacterium* carrying pCAMBIA1300: *TaBBTI-1*$^{-sp}$*-GFP* or pCAMBIA1300: *GFP* constructs was extracted using RIPA Lysis Buffer for Western (Beyotime, China). Equal amount of protein of each sample was separated by 12% SDS-PAGE and transferred into a Polyvinylidene Fluoride (PVDF) membrane. PVDF membrane was blocked with PBST for 5% skim milk powder for 2 h at room temperature and then incubated with anti-GFP polyclonal antibody (1: 1000 dilution) 1 h at room temperature. Anti-actin polyclonal antibody (ABclonal, China) was used as loading control for western blot. The antigen-antibody complexes were visualized using a goat anti-rabbit IgG-conjugated horseradish peroxidase (HRP) antibody (ABclonal, China) at a 1: 10000 dilutions. ECL solution (Bio-Rad, USA) was added to PVDF membrane for visualization, and the chemiluminescence signal was then captured with automatic chemiluminescence image analyzer ChemiDoc MP system (Bio-Rad, USA). Leaves of *N. benthamiana* infiltrated into $MgCl_2$ were used as a blank control. Three replicates were conducted for each treatment.

### 2.12. Prokaryotic expression and purification of TaBBTI-1

The PCR-amplified *TaBBTI-1* coding sequence (*TaBBTI-1*$^{-sp}$) was cloned into the pTOPO-TA/Blunt vector (Aidlab, China) and subcloned into the bacterial expression vector pCZN1 (containing an N-terminal 6 × His-tag). All constructs were verified by bidirectional Sanger sequencing. For recombinant protein expression, a sequence-verified clone was cultured in LB medium at 37 °C until OD600 reached 0.6, followed by induction with 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) at 18 °C for 16 h. Cells were harvested by centrifugation (10,000 ×*g*, 10 min, 4 °C) and resuspended in 20 mL lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM PMSF, supplemented with bacterial protease inhibitor cocktail). After ultrasonication on ice (5 cycles of 10 s pulse/30 s rest at 40% amplitude), the lysate was centrifuged (12,000 ×*g*, 30 min, 4 °C). SDS-PAGE (12%) analysis confirmed target protein accumulation in inclusion bodies.

The inclusion body pellet was solubilized in denaturation buffer (20 mM Tris-HCl, 8 M guanidine hydrochloride, 5 mM DTT, pH 8.0) with gentle agitation for 2 h at 25 °C. The protein solution was dialyzed in 20 mM Tris-HCl and 0.15 M NaCl, pH 8.0 three times for 1 h each, followed by dialysis overnight before purification. Ni-IDA-Sepharose Cl—6B column (Novagen, China) were used to purify the proteins. The column was preequilibrated with Ni-IDA binding-buffer, and the protein solution was allowed to pass through the column and then washed and eluted with Ni-IDA washing-buffer (20 mM Tris-HCl, 20 mM imidazole, 0.5 M NaCl, pH 8.0) and Ni-IDA elution-buffer (20 mM Tris-HCl, 250 mM imidazole, 0.5 M NaCl, pH 8.0). Eluted fractions were dialyzed against PBS (pH 7.4) for 12 h at 4 °C. Protein purity was confirmed by SDS-PAGE (12%), and concentration was determined by Bradford protein assay (Bio-Rad, CA, USA). Aliquots of purified TaBBTI-1 were stored at −20 °C.

### 2.13. Detection of trypsin inhibition activity of TaBBTI-1 in vitro

Trypsin inhibition activity of TaBBTI-1 was determined as previously described [34]. Briefly, 6.25, 12.5, 18.25, and 25 μg of TaBBTI-1 protein (0.5 mg/mL) were added to 50 μL 20 μg/mL of bovine pancreas trypsin (Sigma-Aldrich, USA) dissolved in 50 mM Tris-HCl buffer containing 20 mM calcium chloride (pH 8.0). The mixture was incubated at 37 °C for 15 min. Subsequently, trypsin activity was measured by adding 125 μL of 1 mM N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA; Sigma-Aldrich, USA) substrate, prepared in 50 mM Tris-HCl (pH 8.0) with 20 mM calcium chloride and 1% dimethyl sulfoxide (DMSO). After incubation at 37 °C for 20 min, the reaction was terminated by adding 25 μL of 30% acetic acid. The absorbance at 410 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA, USA). Soybean Bowman-Birk inhibitor (SBBI; Sigma-Aldrich, USA) was included as a positive control. Three replicates were conducted for each treatment.

### 2.14. Effects of feeding on artificial diet containing recombinant TaBBTI-1 on aphid performance

To evaluate the direct inhibitory effects of recombinant TaBBTI-1 on aphid performance, purified TaBBTI-1 protein was added into an artificial diet (20% sucrose solution) at final concentrations of 20 and 100 μg/mL. A 20% sucrose contained the same volume of PBS buffer diet served as the blank control. 200 μL of artificial diet was sandwiched between two layers of Parafilm membrane stretched across a PVC tube (27 mm in diameter, 40 mm high) [32]. Following a 1 h starvation period, five apterous adults *S. avenae* or *M. persicae* were transferred to each feeding device. The number of survived adult aphids and produced nymph were recorded after 5 days feeding. The experiment included twelve biological replicates. The differences among groups were analyzed by one-way ANOVA followed by Duncan's test.

### 2.15. Expression of TaBBTI-1 in wheat leaves and effect on aphid performance

The coding sequence excluding signal peptide of *TaBBTI-1* (*TaBBTI-1*$^{-sp}$) was amplified using FastPfu DNA Polymerase (TransGen Biotech, China) according to the manufacturer's instructions. The primers used are listed in Table S1. The pEDV6: *TaBBTI-1*$^{-sp}$ recombinant plasmids that carry gentamicin resistance were constructed using Gateway technology (Invitrogen, CA, USA) as described previously, and transformed into the *Pseudomonas fluorescens* strain EtHAn that carries chloramphenicol resistance by electroporation [35]. Recombinant strains of EtHAn were grown in KB liquid medium (30 μg/mL gentamicin, 50 μg/mL chloramphenicol) for 48 h and resuspended in 10 mM $MgCl_2$ solution (OD600 = 1.5). EtHAn suspensions were infiltrated into the second leaves of 12-day-old wheat using a 1 mL syringe. The infiltrated plants were grown and maintained in growth chambers under controlled conditions (L: D = 16 h: 8 h; 20 ± 1 °C). After 4 days of infiltration, a wingless adult *S. avenae* were transferred to infiltrated leaves within transparent plastic clip cages (2.7 cm × 2.7 cm × 2.7 cm) to detect the effects of delivery of *TaBBTI-1* into wheat leaves on the performance of grain aphid *S. avenae*. The number of survived aphids and nymphs produced by each aphid was counted for 5 days. The pEDV6: *DsRed* was used as a negative control. A total of twenty replicates were performed for each treatment. The differences between wo treatments were determined using Student's *t*-test.

### 2.16. Performance assays of aphids and cotton bollworms on Nicotiana tabacum plants overexpressing TaBBTI-1

*Agrobacterium* carrying pCAMBIA1300: *TaBBTI-1*$^{-sp}$*-GFP* and pCAMBIA1300: *GFP* constructs were then infiltrated into the leaves of 4-week-old *Nicotiana tabacum* plants as described above. Following 4 days of infiltration, five wingless adult *M. persicae* were transferred onto infiltrated *N. tabacum* leaves. After five days, nymph production per aphid was recorded. The empty vector pCAMBIA1300: *GFP* was used as a negative control. A total of twenty replicates were performed for each treatment. To further assess the effects of *TaBBTI-1* on lepidopteran pest performance, five *H. armigera* second-instar larvae were placed centrally on leaves. Leaf damage was assessed 48 h post-infestation, and lesion areas were quantified using ImageJ software. Six biological replicates were set for each treatment. The differences between two groups were examined using Student's *t*-test.

### 2.17. Statistical analysis

All data were analyzed using IBM-SPSS Statistics 26.0 software (SPSS Inc., Chicago, IL., USA). *P* values $\leq$0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Host preference and feeding behavior of Sitobion avenae, Myzus persicae and Acyrthosiphon pisum on wheat plants

Choice test was conducted to investigate the host plant preferences of three aphid species on wheat when compared to their respective natural host plants. All aphid species exhibited the highest preference to select their natural host plants (Fig. 1A). Also, grain aphid *S. avenae* showed a significantly lower selection rate ($P \leq 0.05$) for tobacco and broad bean compared to wheat. Green peach aphid *M. persicae* displayed the least preference for wheat when compared to tobacco and broad bean ($P \leq 0.05$). Pea aphid *A. pisum* also showed significantly less preference for ($P \leq 0.05$) wheat, a non-host plant, than for broad bean. These suggested that wheat was less preferred as a host for *M. persicae* and *A. pisum*.

Previous studies have reported that continuous aphid feeding on host plants induce $H_2O_2$ and callose accumulation at feeding sites [6,36]. Both *S. avenae* and *M. persicae* infestation were found by DAB and aniline blue assays to induce obvious $H_2O_2$ accumulation and callose deposits in wheat (Fig. 1B, C). In contrast, nothing similar was observed in wheat plants after infested by *A. pisum*. These suggested that *S. avenae* and *M. persicae* engaged in sustained feeding on wheat plants, while *A. pisum* failed to establish effective feeding behaviors. EPG analysis related to these three aphid species on wheat were performed to explore differences in aphid probing and feeding behavior during non−/poor-host versus host interactions. Feeding behavior of *S. avenae, M. persicae and A. pisum* varied greatly when feeding on wheat (Fig. 1D). Grain aphid *S. avenae* showed the longest sustained phloem ingestion (E2 > 10 min) when feeding on wheat plants. During *M. persicae* feeding on wheat, aphids successfully reached phloem, but both the frequency and duration of sustained E2 phase were significantly lower than those of *S. avenae*, while spent significantly longer duration feeding on xylem ($P \leq 0.01$) (Fig. 1E). Total duration of non-probing (np) phase of *A. pisum* feeding on wheat was 2.7- and 1.8- longer than *S. avenae* and *M. persicae*, respectively. Notably, no E2 phase was observed in *A. pisum* when feeding on wheat plants, demonstrating that it failed to reach the phloem for sap uptake.

### 3.2. Aphid fitness on wheat plants

Aphid bioassays were performed to examine the fecundity and honeydew excretion of *S. avenae*, *M. persicae* and *A. pisum* post feeding on wheat plants at 8 and 14 days. *M. persicae* was able to produce nymphs after feeding on wheat, the number of produced nymphs was significantly lower than that of *S. avenae* feeding on wheat plants (Fig. 2A). None of nymphs produced by *A. pisum* were observed at 8 and 14 days. In addition, the amounts of honeydew excreted by *M. persicae* were significantly lower than those of *S. avenae* ($P \leq 0.01$), while no honeydew was produced by *A. pisum* on wheat plants, since *A. pisum* could not feed or survive on wheat (Fig. 2B). The growth performances of *M. persicae* on wheat and natural host tobacco plants were then compared. The number of nymphs and adult weight of *M. persicae* reared on wheat were significantly lower than those on tobacco (Fig. 2C, D). While *M. persicae* demonstrated survival and reproductive ability on wheat, its performances were markedly inferior to *S. avenae*. Based on these results, we classified *M. persicae*-wheat as a poor-host interaction and *A. pisum*-wheat as a non-host interaction.

Due to very limited stylet probing activities and absence of phloem feeding by *A. pisum* on wheat, we primarily compared wheat responses to *S. avenae* and *M. persicae*. The effects of pre-infestation with either first (host interactions) or second aphid species (poor interactions) on subsequent performances on wheat were examined. Also, survival rates of *S. avenae* did not differ significantly after feeding on wheat pre-infested with *S. avenae* or *M. persicae* (Fig. 2E). However, numbers of nymphs produced by *S. avenae* was significantly lower when feeding on *M. persicae*-pre-infested- compared to *S. avenae*-pre-infested-wheats (Fig. 2F).

### 3.3. Comparative transcriptome analysis of wheat host-/ poor-host interactions with Sitobion avenae and Myzus persicae

To further investigate wheat host and poor-host responses to *S. avenae* and *M. persicae*, global transcriptomic changes were examined in wheat plants respectively infested with *S. avenae* and *M. persicae* at 6 hpi and 24 hpi (Table S3). PCA of gene expression levels showed that replicates within the same group clustered closely together, indicating high biological reproducibility. Samples from different time points of *S. avenae* and *M. persicae* infestation clustered distinctly from each other and from controls, demonstrating that aphid feeding induced significant changes in wheat gene expression (Fig. 3A). Compared to uninfested wheat plants, 2619 (1598 upregulated, 1021 downregulated) and 13,726 (7692 upregulated, 6034 downregulated) DEGs were identified in wheat following 6 h and 24 h of feeding by *S. avenae*, respectively (Fig. 3B). After *M. persicae* 6 h and 24 h post-infestation, 2250 (1842 upregulated, 408 downregulated) and 8179 (5239 upregulated, 2940 downregulated) DEGs were identified when compared to controls (Fig. 3B and Table S4-S7).

KEGG enrichment analysis was further performed to explore the biological functions of DEGs in four comparison groups (Fig. 3C and Table S8). The upregulated DEGs in Sa6h vs. 0 h and Sa24h vs. 0 h comparisons were mainly enriched in defense related metabolic pathways including phenylpropanoid, monoterpenoid and tyrosine-tryptophan biosynthesis respectively. While, the upregulated DEGs in the Mp6h vs 0 h and Mp24h vs 0 h comparisons were mainly enriched in alpha-linolenic acid metabolism. The downregulated DEGs induced in both four comparisons were mainly enriched in photosynthesis-antenna proteins and carotenoid biosynthesis (Fig. 3C). GO enrichment analysis was conducted to identify the functions of all identified DEGs (Fig. S1 and Table S9). In the Sa6h vs 0 h and Sa24h vs 0 h comparisons, within the biological process category, the upregulated DEGs were mainly enriched in “response to light stimulus” and “translation”. The largest proportion of upregulated DEGs induced by *S. avenae* feeding was enriched in “DNA-binding transcription factor activity”, “calcium ion binding”, “carbohydrate binding” and “structural constituent of ribosome” within the molecular function category (Fig. S1). In the Mp6h vs 0 h and Mp24h vs 0 h comparisons, within the biological process category, the upregulated DEGs were mainly enriched in response to wounding”, “protein folding”, “oxylipin biosynthetic process” and “regulation of jasmonic acid mediated signaling pathway”; and within

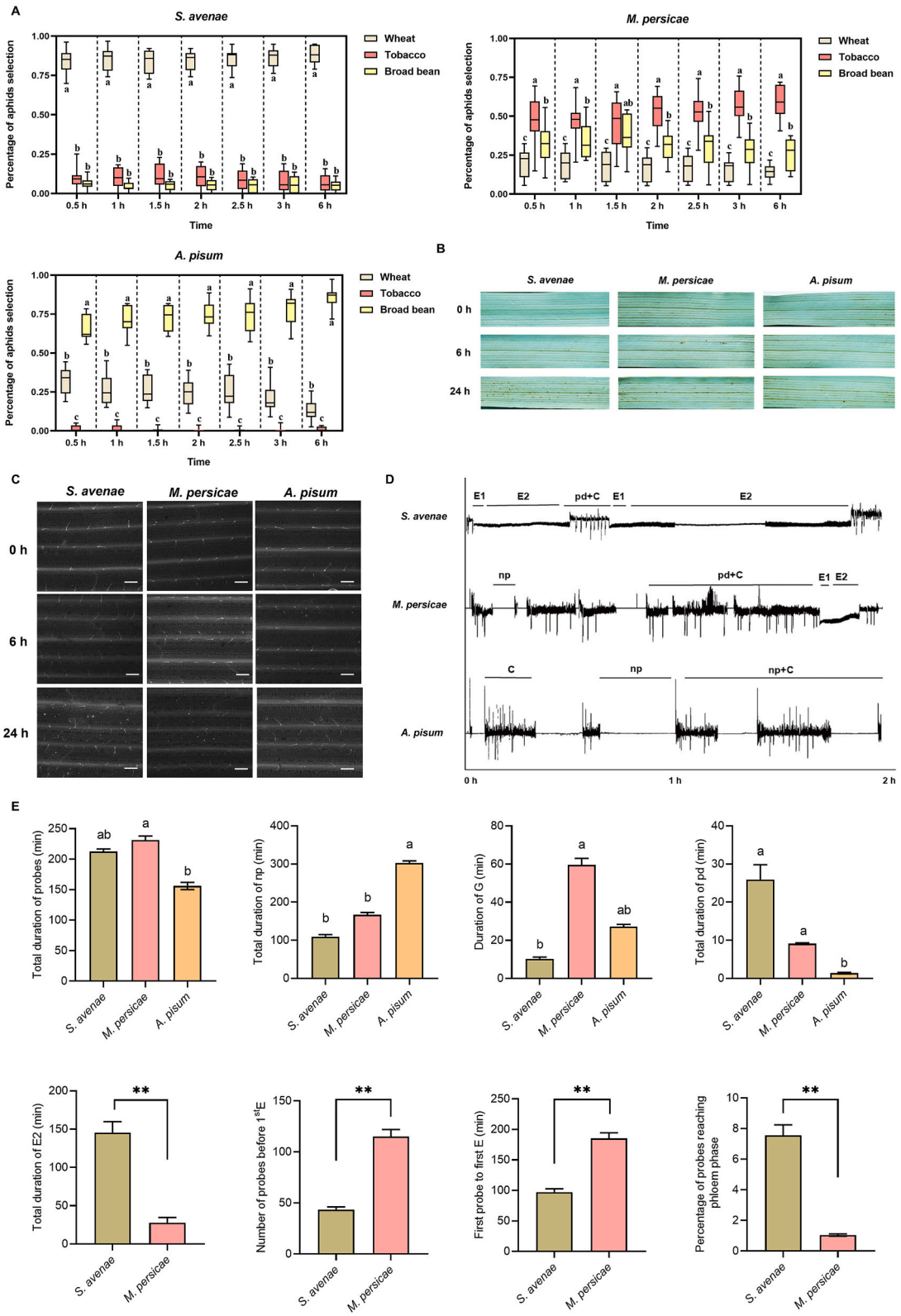


**Fig. 1.** Host preference and feeding behaviors of *Sitobion avenae*, *Myzus persicae*, and *Acyrthosiphon pisum* on wheat. (A) Host selection percentages of each aphid species on wheat, tobacco and broad bean plants. Data points and error bars represent means ± standard error ($n = 10$). Different lowercase letters indicate significant differences among groups ($P \leq 0.05$). (B-C) Hydrogen peroxide accumulation and callose deposition in wheat leaves infested with *S. avenae*, *M. persicae* and *A. pisum* at 0 h, 6 h, and 24 h. (D) Representative electrical penetration graph waveforms of each aphid species feeding on wheat plants. np: no probing; C: stylet probing; E1: salivation; E2: phloem ingestion. (E) Feeding behavior parameters of *S. avenae*, *M. persicae* and *A. pisum* feeding on wheat plants. Data are presented as mean ± standard error ($n = 15$). Different lowercase letters represent significant differences among different groups One-way ANOVA test followed by Duncan's multiple comparisons. ($P \leq 0.05$). Asterisks above the bar indicated significant differences between different groups (**$P \leq 0.01$).

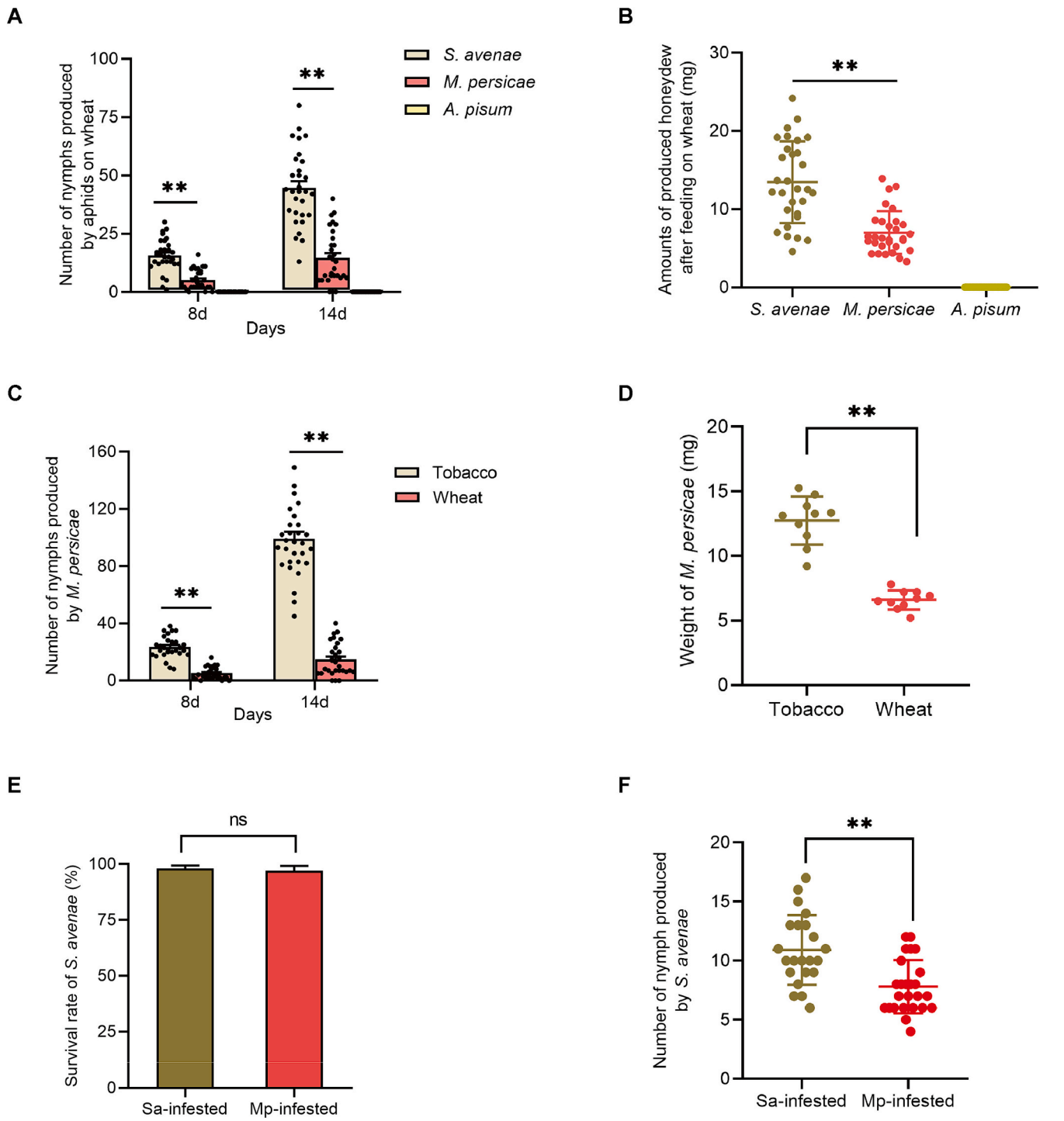


**Fig. 2.** Performance of *Sitobion avenae*, *Myzus persicae*, and *Acyrthosiphon pisum* on wheat. (A) Number of nymphs produced by each aphid species after feeding on wheat plants for 8 and 14 days ($n = 30$). (B) Amounts of honeydew excreted by *S. avenae*, *M. persicae* and *A. pisum* after feeding on wheat for 48 h (n = 30). (C) Number of produced nymphs after *M. persicae* fed on tobacco and wheat for 8 and 14 days (n = 30). (D) Weight of *M. persicae* after14 days of feeding on tobacco and wheat (n = 10, Student's *t*-test). (E) Survival rate of *S. avenae* after feeding on wheat plants pre-infested with *S. avenae* or *M. persica* for 5 days ($n = 20$). (F) Number of nymphs produced by *S. avenae* after feeding on *S. avenae*-preinfested or *M. persicae*-preinfected wheat for 5 days (n = 20). Data are presented as mean ± standard error. Asterisks above the bar indicated significant differences between different groups (**$P \leq 0.01$).

the molecular function category, the upregulated DEGs were mainly enriched in "DNA-binding transcription factor activity", "unfolded protein binding" and "terpene synthase activity" (Fig. S1).

To identify *M. persicae*-specific transcriptional responses, we performed comparative analysis of upregulated DEGs in aphid-infested versus non-infested wheat plants using Venn diagrams. In the poor-host interaction, *M. persicae* feeding specifically upregulated 754 and 1008 genes in the Mp6h vs 0 h and Mp24h vs 0 h comparisons, respectively. A comparison of wheat DEGs induced by *M. persicae* and *S. avenae* identified a core set of 101 genes that were significantly upregulated in both Mp6h vs Sa6h and Mp24h vs Sa24h comparisons (Fig. 3D; Tables S10, S11). Among these, a Bowman-Birk trypsin inhibitor (TraesCS3D02G034200), which were designated as TaBBTI-1, showed significant upregulation in both comparison groups (Fig. 3D).

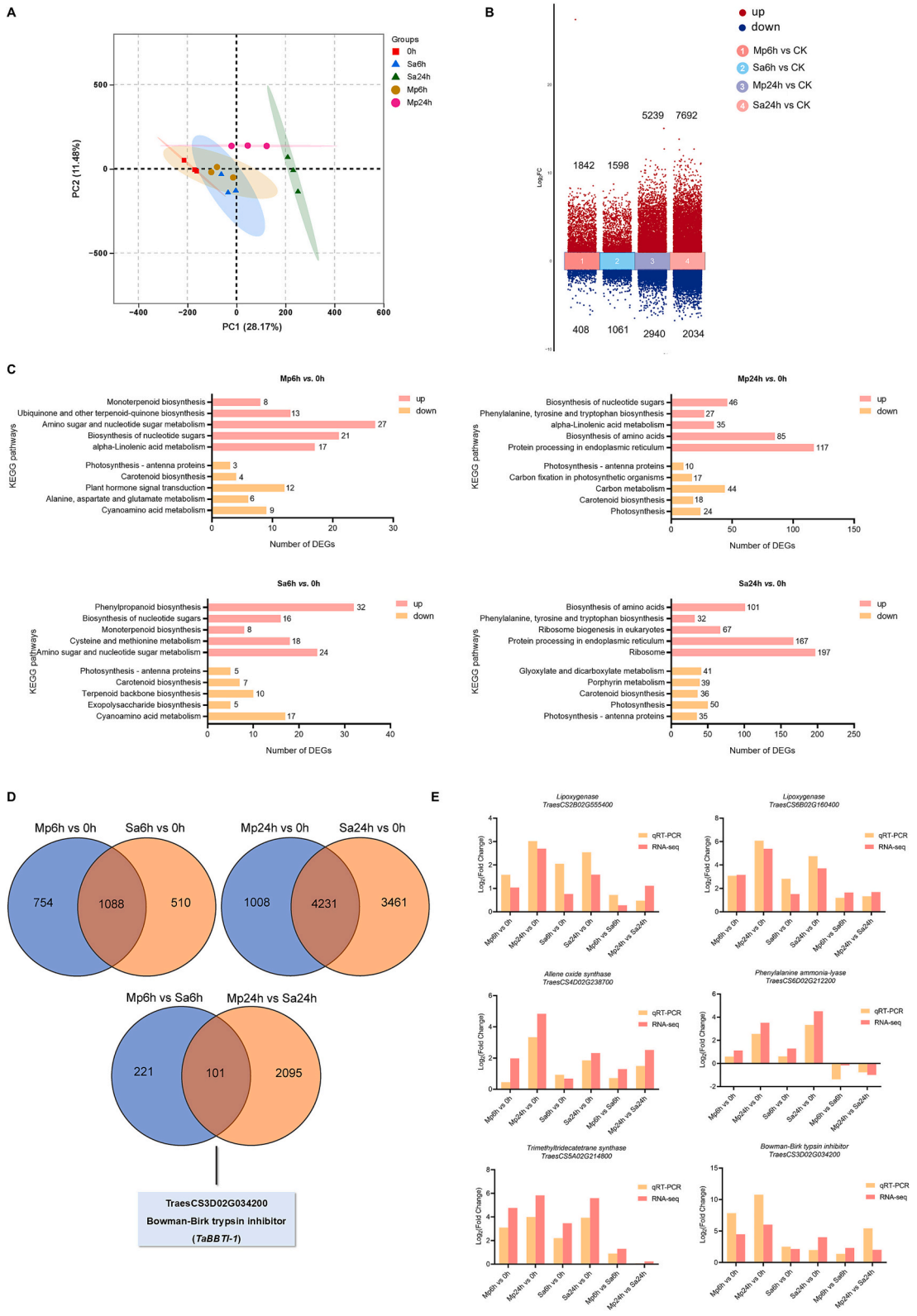


**Fig. 3.** Wheat transcriptome profiling in response to interactions with *Sitobion avenae* versus interaction with *Myzus persicae*. (A) Principal component analysis (PCA) of the expression of unigenes for 0 h, Sa6h, Sa24h, Mp6h, and Mp24h. (B) Number of upregulated and downregulated DEGs in different comparison groups. (C) KEGG enrichment analysis of the DEGs in Sa6h vs 0 h, Sa24h vs 0 h, Mp6h vs 0 h, Mp24h vs 0 h comparison groups. (D) Venn diagrams showing the overlap between upregulated DEGs in Sa6h vs 0 h, Sa24h vs 0 h, Mp6h vs 0 h, Mp24h vs 0 h, Mp6h vs Sa6h, and Mp24h vs Sa24h comparison groups. (E) Comparison of expression levels of key genes in RNA-seq and qRT-PCR analysis. Yellow bar represents the fold change in relative gene expression determined by quantitative real-time PCR, and red bar represents the fold change in relative gene expression determined by transcriptome data.

To verify the transcriptome sequencing results, six genes were selected to examine the changes of expression levels in wheat infested with *S. avenae* and *M. persicae* at 6 h and 24 h. These genes included *TaLOX* (TraesCS6B02G160400, TraesCS2B02G555400), *TaAOS* (TraesCS4A02G238700), *TaPAL* (TraesCS6D02G212200), trimethyltridecatetrane synthase (*TaTMTT*, TraesCS5A02G214800), and *TaBBTI-1* (TraesCS3D02G034200). Changes in the expression levels of six genes were highly consistent with the transcriptome sequencing results, indicating the validity of RNA-seq results (Fig. 3E).

### 3.4. Effect of Myzus persicae infestation on wheat induced jasmonic acid signaling defense pathway during poor-host interactions

We systematically analyzed DEGs associated with SA and JA signaling pathways given the well-established roles of these pathways in plant defense against herbivory. In SA pathway response, several *PAL* genes showed significantly upregulation in wheat infested by *S. avenae* or *M. persicae* compared with the controls (Table S4-S7). A total of 42 JA-associated DEGs, including *LOX*, *AOS*, *Allene oxide cyclase* (*AOC*), *12-oxo-phytodienoic acid reductase* (*OPR*), *OPC-8: 0 CoA ligase 1* (*OPCL1*); *acyl coenzyme A oxidase* (*ACX*), and *multifunctional protein* (*MFP*), were identified in all groups (Fig. 4A). Of these, 17, 42, 8, and 31 JA-responsive genes were significantly regulated at Mp6h vs. 0 h, Mp24h vs. 0 h, Sa6h vs. 0 h and Sa24h vs. 0 h comparison group, respectively. In addition, *M. persicae* induced significantly higher expression levels of JA-responsive genes (*LOX*, *AOS*, *AOC*, *OPR*, *OPCL1*, *ACX*, and *MFP*) compared to *S. avenae*. For example, the expression of *LOX* (TraesCS6A02G132500), *AOS* (TraesCS4A02G061900), and *AOC* (TraesCS6B02G365200) was upregulated approximately 51-, 62-, and 18-fold in the Mp24h vs 0 h comparison, while the transcript levels of those genes were upregulated 17-, 31-, and 11-fold in the Sa24h vs 0 h (Table S5, S7).

The contents of SA and JA in wheat leaves following infestation by *S. avenae* and *M. persicae* were then quantified using LC-MS/MS. We found that feeding by *M. persicae* induced a significant increase in JA content, whereas feeding by *S. avenae* on wheat shows no significant difference in JA content compared to non-infested controls ($P \leq 0.05$; Fig. 4B). While, both *S. avenae* and *M. persicae* infestation significantly increased SA accumulation in wheat at 6 h and 24 h post-infestation, with no significant differences observed between these two aphid species ($P \leq 0.05$; Fig. 4C).

### 3.5. Effect of exogenous application of jasmonic acid on wheat induced the expression of TaBBTI-1

To elucidate the potential relationships between JA signaling pathway with *TaBBTI-1* expressions induced by *M. persicae* in poor-host interactions, wheat seedlings were exogenously treated with 0.2 mM, 0.5 mM, or 1.0 mM JA for 6 h and 24 h. RT-qPCR analysis revealed that transcript levels of JA pathway-associated genes, including *TaLOX*, *TaFAD*, and *TaAOS* were significantly upregulated ($P \leq 0.05$) across all JA concentrations at both timepoints (Fig. 5), indicating that exogenous application of JA on wheat activated JA signaling defense responses. In addition, we found that 0.2 mM, 0.5 mM, and 1.0 mM of JA significantly induced *TaBBTI-1* expression in wheat seedlings. At 24 h post-treatment, seedlings sprayed with 0.2 mM JA exhibited peak *TaBBTI-1* expression, showing an approximately 104.94-fold increase compared to untreated controls (Fig. 5), suggesting that JA signaling pathway is involved in the induction of *TaBBTI-1*.

### 3.6. TaBBTI-1 as a functional trypsin inhibitor

Sequence analysis showed that *TaBBTI-1* encodes a 176-amino acid protein containing an N-terminal signal peptide, a transmembrane domain, and two Bowman-Birk inhibitor domains (Fig. 6A). The 3D diagram showed that the secondary structure of TaBBTI-1 consists of one α-helix, eight $\beta$-sheets and loops (Fig. 6B). Phylogenetic analysis classified 44 wheat BBTI proteins into seven distinct clades, with TaBBTI-1 (TraesCS3D02G034200) clustering with ten genes and showing the closest phylogenetic relationships with TraesCS3A02G04600 (Fig. 6C). Subcellular localization of *TaBBTI-1* in the leaves of *N. benthamiana* revealed that *TaBBTI-1*$^{-sp}$*-GFP* fusion constructs exhibited fluorescence throughout cytoplasm and nucleus, indicating cytoplasmic and nuclear localization of *TaBBTI-1* (Fig. 6D). Western blot analysis confirmed successful heterologous expression, with GFP-Flag fusion protein detected in both *TaBBTI-1*$^{-sp}$*-GFP*- and *GFP*-expressing leaves, while absent in mock controls (Fig. 6E).

To verify the trypsin inhibitor activity of TaBBTI-1, the protein was heterologous expressed in *Escherichia coli* using a prokaryotic expression system. SDS-PAGE and western blot analysis showed that TaBBTI-1 was successfully purified, exhibiting a molecular weight of approximately 17 kDa (Fig. 6F, G). Functional analysis by trypsin inhibition assay demonstrated dose-dependent inhibitory activity that trypsin activity decreased progressively with increasing TaBBTI-1 concentrations (Fig. 6H), exhibiting inhibition comparable to the positive control soybean Bowman-Birk inhibitor (SBBI). These results established TaBBTI-1 as a functional Bowman-Birk-type trypsin inhibitor.

### 3.7. Effect of TaBBTI-1 on plant resistance against aphids and cotton bollworm

To determine whether TaBBTI-1 affects aphid performance, the effects of direct feeding on artificial diets containing different concentrations of purified TaBBTI-1 protein on the survival and fecundity of *S. avenae* and *M. persicae* were examined. Feeding on an artificial diet supplemented with 20 μg/mL TaBBTI-1 significantly decreased the number of nymphs produced by both aphid species when compared with controls ($P \leq 0.05$). The number of nymphs produced by *S. avenae* gradually reduced as TaBBTI-1 protein concentrations increased to 100 μg/mL (Fig. 7A). When feeding on 20 μg/mL TaBBTI-1 protein, the number of aphids produced by each *S. avenae* adult over five days reduced to 5.03, and further decreased to 3.08 after feeding on artificial diet with 100 μg/mL TaBBTI-1 protein, which was significantly lower than that produced by controls ($P \leq 0.05$). However, no significant differences were observed in the survival rates of *S. avenae* and *M. persicae* after feeding on TaBBTI-1 protein.

Effects on aphid performance following feeding on wheat and tobacco plants transiently overexpressing *TaBBTI-1* were also assessed to further validate the resistance traits of the wheat TaBBTI-1 protein against insect pests. Number of nymphs produced by *S. avenae* on wheat leaves delivered with *TaBBTI-1* via the type III secretion system of *P. fluorescens* EtAnH was significantly lower than control groups (Fig. 7B). Similarly, the number of produced nymphs by *M. persiace* was only 15 after feeding on leaves transiently overexpressing *TaBBTI-1*, with a reduction of nearly 60% compared with that of feeding on leaf areas infiltrated with the *Agrobacterium* strain carrying the vector control (Fig. 7C). *TaBBTI-1* was then transient overexpressed in *M. persicae* host plant *N. tabacum* mediated by *A. tumefaciens*, and western blot analysis confirmed *TaBBTI-1* was successful ectopically expressed in plant leaves (Fig. 7D). The feeding preference of polyphagous lepidopteran pest, namely cotton bollworm *Helicoverpa armigera* on *TaBBTI-1*-overexpressed *N. tabacum* was also investigated. The preferential feeding assays revealed that *H. armigera* preferred to feed on control plants compared with *TaBBTI-1*-overexpressed *N. tabacum* leaves (Fig. 7E). The results of the leaf area after feeding damages also showed that the *H. armigera* larvae fed on approximately $3.49 \pm 0.73$ cm$^2$ area of the control leaves expressing *GFP*. However, the damaged area of the leaves overexpressed *TaBBTI-1* by *H. armigera* larvae feeding was only $1.32 \pm 0.22$ cm$^2$, which was significantly lower than that of control groups ($P \leq 0.05$) (Fig. 7F). These results demonstrated that TaBBTI-1 confers broad-spectrum resistance of plants against aphids and lepidopterans.

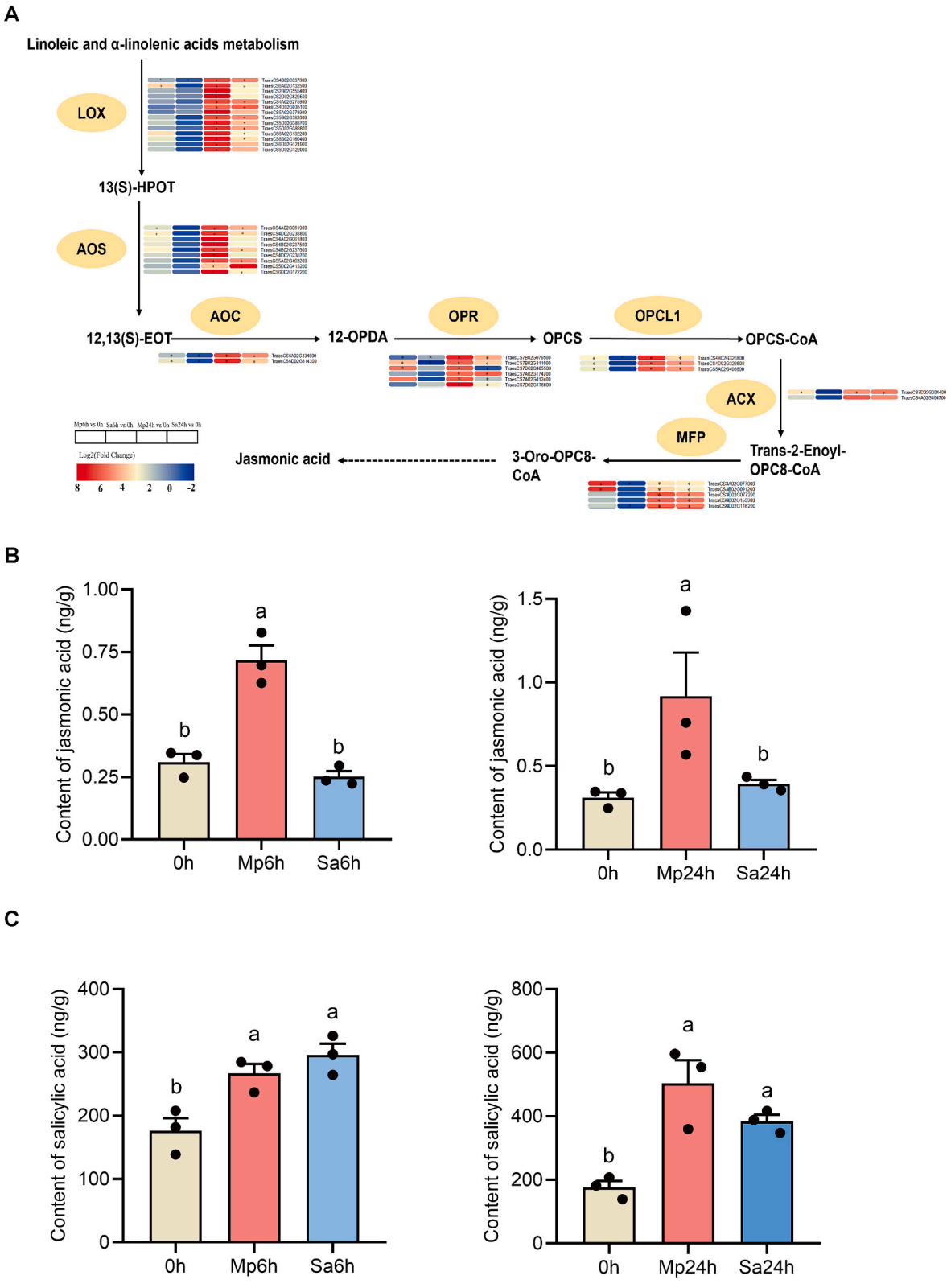


**Fig. 4.** Induced jasmonic acid signaling defense pathway in wheat following *Myzus persicae* infestation. (A) Expression profiles of DEGs involved in jasmonic acid biosynthesis. Heatmap shows $Log_2$ (fold change) values for Mp6h vs 0 h, Sa6h vs 0 h, Mp24h vs 0 h, and Sa24h vs 0 h comparisons. Ellipses represent individual genes. Asterisks (*) indicate significant differential expression ($P \leq 0.05$). Colour scale ranges from −2 to 8. *LOX*: *Lipoxygenase*; *AOS*: *Allene oxide synthase*; *AOC*: *Allene oxide cyclase*; *OPR*: *12-oxo-phytodienoic acid reductase*; *OPCL1*: *OPC-8:0 CoA ligase 1*; *ACX*: *Acyl coenzyme A oxidase*; *MFP*: *Multifunctional protein*. Endogenous jasmonic acid (B) and salicylic acid (C) levels in wheat leaves infested with *S. avenae* or *M. persicae* at 6 h and 24 h post-infestation. Three replicates were conducted for each treatment. Data are presented as mean ± standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$).

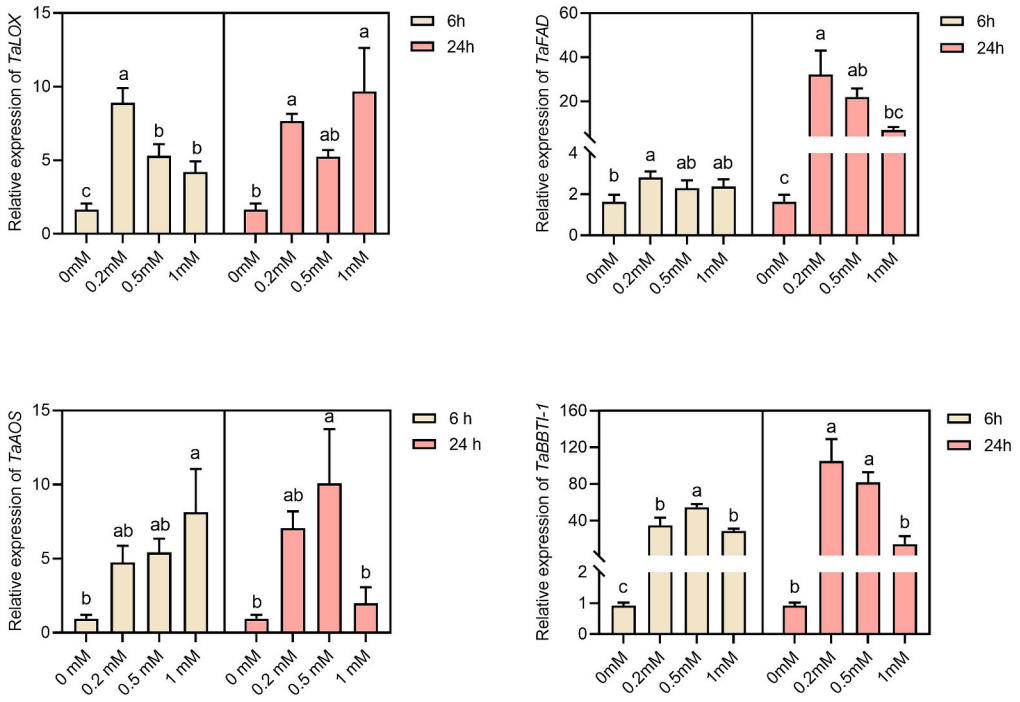


**Fig. 5.** Impact of exogenous application of jasmonic acid on gene expression levels. Effects of jasmonic acid treatment on the expression of *TaLOX*, *TaFAD*, *TaAOS* and *TaBBTI-1* in wheat. Data are presented as mean ± standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$).

## 4. Discussion

Non-host plant resistance, typically defined as the resistance of plants to incompatible microbial pathogens [37], is a well-established concept in plant-pathogen interactions. However, its occurrence and underlying mechanisms remain rarely reported in the context of plant-insect interactions. As important agricultural insect pests, most aphid species are oligophagous specializing on a limited range of hosts, whereas a small number are polyphagous and exhibit remarkable adaptability to the nutritional quality and defense responses of diverse host plants [38]. Understanding how plants differentially respond to aphids with varying host ranges including potential non-/poor-host resistance mechanisms and identifying the underlying responsive signaling pathways are essential for developing novel and durable aphid management strategies.

In this study, we characterized interactions between wheat and three aphid species *S. avenae*, *M. persicae*, and *A. pisum* using adaptability assays. Our results demonstrated distinct responses, with *S. avenae* exhibiting the strongest preference for and growth performance on wheat. *M. persicae* could feed on wheat, a non-natural host, ingesting phloem sap but showing reduced fecundity. Conversely, *A. pisum* was unable to feed on or survive on wheat. Although *M. persicae* and *A. pisum* have not been reported to cause significant infestations on wheat, *M. persicae* has been found to colonize winter wheat, indicating that this aphid is able to survive on this crop [20]. A previous study has also shown that *M. persicae* can survive and reproduce on the cereal crop barley but perform poorly on this host compared with the well-documented host plant oilseed rape (*Brassica napus*) [21]. Aphid probing behavior reflects the adaptability of insect pests to plants during the initial penetration of stylets into plant tissues [39,40]. According to our EPG assays, *M. persicae* stylets could reach the xylem, exhibiting longer xylem phases (G waveform), but phloem ingestion was inhibited. Extended G waveforms are interpreted as a compensatory strategy to maintain water balance when phloem uptake is limited during incompatible interactions [41]. A previous study also demonstrated that aphids spent more time feeding on the xylem but less time feeding on the phloem on the aphid-resistant variety Ww2730 compared with the aphid-susceptible variety Batis [42]. This pattern of reduced phloem access and prolonged xylem feeding confirms wheat as a poor host for *M. persicae*. For *A. pisum*, representing a non-host interaction, stylets failed to reach the phloem, with resistance likely localized in the mesophyll. Frequent brief probing behavior by aphids on plants has also been shown to be a characteristic of non-host interactions [43,44]. A feature of the *A. pisum*-wheat non-host interaction was an increased total number and duration of probes, as well as longer no-probing periods, which is similar to *R. padi-Arabidopsis* non-host interactions [45]. Furthermore, previous studies have reported that continuous aphid feeding induces $H_2O_2$ and callose accumulation in plants during both compatible and incompatible interactions [46,47]. Evident $H_2O_2$ accumulation and callose deposition occurred at stylet penetration sites in epidermal cells during both *S. avenae* and *M. persicae* interactions, signifying active molecular defense responses in these host/poor-host interactions. Conversely, no $H_2O_2$ or callose accumulation was detected in *A. pisum*-infested leaves, consistent with its limited probing and failed feeding, reflecting the absence of sustained interactions that trigger these defenses.

Interestingly, we observed that the fecundity of *S. avenae* feeding on wheat pre-infested with *M. persicae* was significantly reduced compared with that of aphids feeding on wheat pre-infested with *S. avenae* itself. This finding suggests that *M. persicae* elicits a stronger defense response in wheat during its poor-host interaction. According to comparative transcriptomic analyses, infestation by both *S. avenae* and *M. persicae* induced significant expression changes in >2000 wheat genes within 6 h, indicating rapid activation of wheat defense pathways against aphid feeding However, wheat infested with *M. persicae* exhibited a higher number of upregulated DEGs than that infested with *S. avenae*, further demonstrating the enhanced early physiological response triggered by the former aphid species. A previous study has shown that host and non-host aphid interactions induce distinct but partially overlapping DEG patterns [18]. Specifically, we identified 1088 and 4231 co-upregulated DEGs in both interactions at early and short-term time points, respectively. More importantly, *M. persicae* infestation specifically upregulated fewer but significant genes, whereas *S. avenae* specifically upregulated a great number of genes at similar infestation durations (1008 and 3461

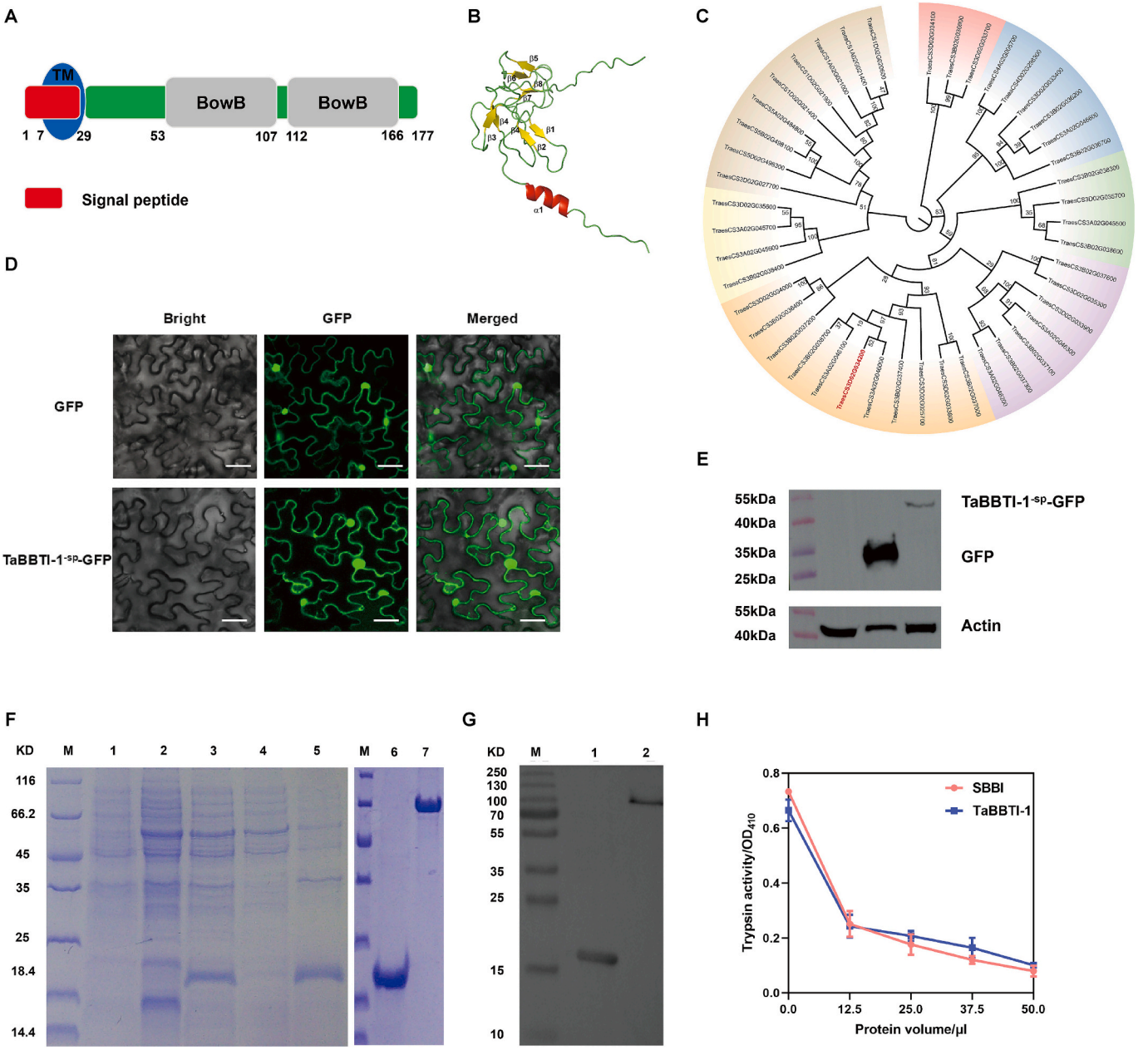


**Fig. 6.** Biochemical characterization of TaBBTI-1. (A) Sequence analysis of amino acid sequence of TaBBTI-1. The predicted of the signal peptide, transmembrane domain and two BowB domains are indicated. (B) 3D structural model of TaBBTI-1. (C) Phylogenetic tree constructed by comparing the amino acid sequences of 44 TaBBTI-1 proteins from wheat. The phylogenetic tree was constructed by the neighbor-joining method using MEGA7.0. Bootstrap values calculated as a percentage for 1000 replications are shown at the nodes. (D) Subcellular localization of *TaBBTI-1* in *N. benthamiana*. *TaBBTI-1*$^{sp}$*-GFP* or *GFP* were transiently overexpressed in *N. benthamiana* leaves by *A. tumefaciens*-mediated infiltration. The images were taken 36 h after agroinfiltration using confocal microscopy. Bar = 20 μm. (E) Western blot analysis of *TaBBTI-1*$^{sp}$*-GFP* in *N. benthamiana* using anti-GFP. Lane 1: Total soluble proteins extracted from empty GV3101 infiltrated leaves; Lane 2: Proteins extracted from leaves infiltrated with *GFP*; Lane 3: Proteins extracted from leaves infiltrated with *TaBBTI-1*$^{sp}$*-GFP*. Blotting with plant anti-actin antibody served as a loading control. (F) SDS-PAGE analysis of TaBBTI-1 protein expression and purification. The TaBBTI-1 protein expressed and purified using Ni-IDA-Sepharose CL-6B was electrophoresed on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. M: Protein molecular weight markers; Lane 1: uninduced pCZN1; Lane 2: uninduced *TaBBTI-1*-pCZN1; Lane 3: induced *TaBBTI-1*-pCZN1; Lane 4: induced TaBBTI-1 supernatant lysate; Lane 5: induced TaBBTI-1 inclusion body; Lane 6: purified protein; Lane 7: 0.5 mg/mL BSA. (G) Western blot analysis of purified TaBBTI-1. M: Protein molecular marker; Lane 1: Purified TaBBTI-1; Lane 2: Multitag protein. (H) Trypsin inhibitor activity of TaBBTI-1 in vitro. The x-axis indicates the protein amount. The y-axis indicates the absorbance of p-nitroaniline at 410 nm. Soybean Bowman-Birk inhibitor (SBBI) was used as the positive control. Data points and error bars represent means ± standard error (n = 3).

genes, respectively). KEGG analysis showed that DEGs in the *M. persicae*-infested treatment were predominantly enriched in alpha-linolenic acid metabolism. This effect was not observed with *S. avenae* infestation. As alpha-linolenic acid is the precursor for JA biosynthesis, and its accumulation promotes JA production and JA-mediated defenses [48,49], this enrichment strongly indicates that JA biosynthesis is specifically stimulated during poor-host interactions with *M. persicae*.

The phytohormones SA and JA are major regulators of plant immunity and confer resistance against aphid attacks [3,25,50]. Consistent with this, we found that endogenous SA levels in wheat leaves increased significantly following infestation by both *M. persicae* and *S. avenae*. Previous studies have also shown that infestation by *S. graminum* and

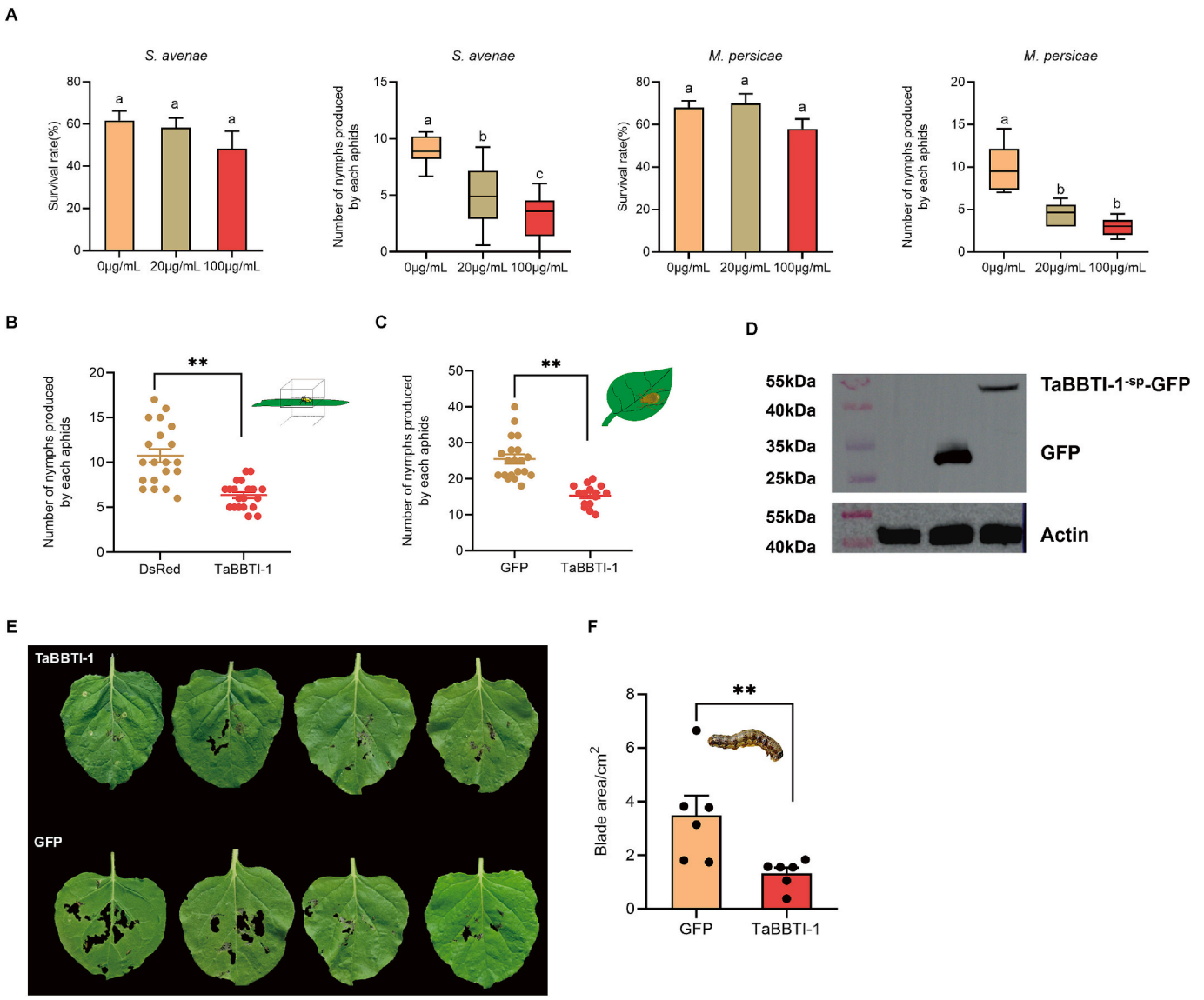


**Fig. 7.** Functional characterization of TaBBTI-1 protein in plant resistance against insect herbivores. (A) Survival rate and fecundity of *S. avenae* and *M. persicae* after feeding on artificial diet supplemented with purified TaBBTI-1 protein. Data are presented as mean ± standard error (n = 10). Different lowercase letters represent significant differences among different groups ($P \leq 0.05$). (B) Number of produced nymphs by *S. avenae* after feeding on wheat leaves inoculated with pEDV6: *TaBBTI-1* or pEDV6: *DsRed* (control) for 5 days (n = 20). (C) Number of produced nymphs by *M. persicae* on *N. tabacum* leaves after infiltrated with recombinant strains of *A. tumefaciens* cells carrying *TaBBTI-1-GFP* or *GFP* for 5 days (n = 20). (D) Western blot detection of *TaBBTI-1* expression in *N. tabacum* leaves inoculated with *A. tumefaciens* carrying *TaBBTI-1-GFP* at two days. Lane 1: Total soluble proteins extracted from leaves infiltrated empty GV3101; Lane 2: Proteins extracted from leaves infiltrated with *GFP*; Lane 3: Proteins extracted from leaves infiltrated with *TaBBTI-1-GFP*. Blotting with plant anti-actin antibody served as a loading control. (*E*-F) Feeding assays of *H. armigera* on *N. tabacum* leaves overexpressing *TaBBTI-1-GFP* or *GFP*. Photographs of leaf damage were taken at two days of feeding. Feeding damage to the leaf tissues were measured using ImageJ software. Data are presented as mean ± standard error ($n = 6$). Asterisks above the bar indicated significant differences between different groups ($^{**}P \leq 0.01$).

*S. avenae* induces SA pathway genes (e.g., *PAL*, *PR1*) and elevates SA concentrations in wheat [6,7,32]. Furthermore, exogenous SA application enhances resistance in aphid-susceptible wheat against *S. avenae* [51]. Although a clear antagonistic relationship exists between SA and JA signaling pathways, JA accumulation can also be induced in resistant maize varieties infested by the sugarcane aphid *Melanaphis sacchari* [49] and in tea plants fed upon by the citrus black aphid *Toxoptera aurantii* [52]. Notably, the JA pathway is considered particularly effective against aphids compared with the SA defense pathway [25]. Exogenous JA application suppresses aphid populations on tomato and *Brassica* species [53,54], whereas inhibiting JA accumulation by silencing *SbLOX3* enhances *M. sacchari* performance on host plants [55]. Our key finding is that in the poor-host interaction (*M. persicae*-wheat), endogenous JA content in wheat plants increased significantly, whereas no significant change occurred following *S. avenae* infestation. This indicates that the JA signaling pathway plays a vital role in wheat resistance specifically during poor-host aphid interactions. This specialized JA activation mirrors its documented role in defense against non-adapted pathogens, such as the non-host resistance of *Arabidopsis thaliana* to barley powdery mildew *Blumeria graminis* f. sp. *hordei* (Bgh) [56] and of sweet orange *Citrus sinensis* to citrus canker caused by *Xanthomonas citri* [57].

Specific genes contributing to plant immunity have been identified in poor- or non-host interactions with different aphid species. For instance, the black cherry aphid *Myzus cerasi* (a poor-host interaction on *Arabidopsis*) exhibited larger populations on *AtRbohF-3* knockout lines than on wild-type controls, demonstrating that the NADPH oxidase *AtRbohF* contributes to non-host defenses [18]. Additionally, transcriptome

analysis of barley infested with *M. persicae* allowed the identification of two *thionin* genes, involved in plant resistance during poor host interactions [21]. Similarly, our transcriptomic analysis revealed that *M. persicae* feeding specifically induced the upregulation of *TaBBTI-1* in wheat during poor-host interactions. BBTIs are serine-type PIs that inhibit trypsin and chymotrypsin activity [58]. The induction of PIs such as BBTIs is a well-documented plant defense mechanism against herbivores. For example, rice infested by the rice stem borer *Chilo suppressalis* shows increased accumulation of trypsin inhibitors [59]. Feeding by brown planthopper *Nilaparvata lugens* nymphs induces the upregulation of PI gene expression in resistant rice plants [60]. Volatiles emitted by rice plants infested with *C. suppressalis* significantly enhance *BBTI* expression and the accumulation of trypsin inhibitors [61]. Previous studies have also demonstrated that methyl jasmonate application induces proteinase inhibitor synthesis in tomato plants [62]. Silencing *OsHI-LOX* significantly reduced both JA and trypsin PI levels in rice, whereas JA treatment increased these inhibitor levels [5]. In the present study, spraying JA on wheat significantly upregulated *TaBBTI-1* levels, indicating that JA-mediated signaling pathways positively regulate TaBBTI-1 accumulation in wheat during poor-host interactions with *M. persicae*; however, the underlying mechanisms remain to be elucidated.

Previous studies have suggested that overexpression of *BBTI* genes can confer resistance to pathogen and insect in crops. For example, overexpression of a cowpea trypsin inhibitor gene encoding a BBTI protein conferred resistance to coleopteran and lepidopteran insects in tobacco [63]. Addition of SbBBTI, derived from soybean, to an artificial diet significantly reduced population of the potato aphid *M. euphorbiae* [64]. Adding purified BBTI Oh43 to insect diets reduced larval weight and inhibited growth in the fall armyworm *Spodoptera frugiperda* [65]. Rice overexpressing *APIP4*, a rice *BBTI*, showed increased resistance to the rice blast fungus *Magnaporthe oryzae* and the stem borer *C. suppressalis*, whereas *APIP4* knockout enhanced host susceptibility [34,66]. Similarly, we demonstrated that purified TaBBTI-1 protein exhibits trypsin inhibitory activity in vitro. Supplementing artificial diets with purified TaBBTI-1 or delivering *TaBBTI-1* to wheat leaves significantly reduced *S. avenae* fecundity. These findings indicate that *M. persicae*-induced upregulation of *TaBBTI-1* during poor-host interactions contributes substantially to induced wheat resistance against aphids. Furthermore, we observed that both *M. persicae* and the lepidopteran pest *H. armigera* exhibited reduced performance on tobacco plants overexpressing *TaBBTI-1*, suggesting that *TaBBTI-1* confers broad-spectrum resistance in plants against both phloem-feeding aphids and chewing insects.

During aphid probing and feeding, elicitors present in secreted saliva can be recognized by plant pattern-recognition receptors, resulting in activation of plant defense responses [67–69]. Several salivary elicitors in aphid saliva such as Mp10, CathB3, and SmCSP4, have been identified as inducers of effective host plant defenses [31,70,71]. Aphids also secrete saliva containing effector proteins into the plant cytoplasm to suppress plant immunity and facilitate colonization of host plants. Aphid effectors from *A. pisum*, *M. persicae* and *M. euphorbiae*, including ApC002, Mp55, Mp1, MpPInt02, and Me23, have been demonstrated to be involved in inhibiting plant defense responses [68,72,73]. The salivary effector Sg2204 of the greenbug *S. graminum* has been shown to suppress both SA- and JA- signaling defense pathways in wheat [35]. A previous study demonstrated that the *M. oryzae* effector AvrPiz-t can directly interact with a BBTI protein in rice, APIP4, thereby suppressing APIP4 trypsin inhibitor activity [34]. In total, 71 and 114 salivary proteins have been identified in *M. persicae* and *S. avenae* saliva, respectively; these include several species-specific proteins, with ABC transporters uniquely detected in *S. avenae* [74,75]. Whether these diverse salivary proteins from *M. persicae* and *S. avenae* specifically modulate the JA-mediated induction of *TaBBTI-1* warrants further investigation.

In conclusion, our study demonstrated that *M. persicae* infestation (poor-host interactions) triggers stronger wheat resistance than *S. avenae* infestation (host interactions), as evidenced by reduced aphid fecundity on *M. persicae*-preinfested wheat plants. Transcriptomic analysis revealed that the JA signaling pathway mediates wheat defense responses to *M. persicae*. Specifically, infestation by *M. persicae*, but not *S. avenae* significantly elevated endogenous JA levels, thereby inducing expression of TaBBTI-1 in wheat during poor-host interactions. Functional validation confirmed that feeding recombinant TaBBTI-1 protein or using wheat plants overexpressing *TaBBTI-1* impaired *S. avenae* fecundity and that ectopic expression in *N. tabacum* conferred broad-spectrum resistance against both *M. persicae* and *H. armigera*. This JA-TaBBTI-1 module represents a novel mechanism underlying poor-host resistance in wheat, providing a genetic basis for improving broad-spectrum crop resistance against diverse pests.

## CRediT authorship contribution statement

**Xiaobei Liu:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Yumeng Zhang:** Validation. **Frédéric Francis:** Writing – review & editing. **Yumeng Cheng:** Data curation. **Yanxia Liu:** Resources, Conceptualization. **Julian Chen:** Writing – review & editing, Conceptualization. **Yong Zhang:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

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## Declaration of competing interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2026.150204.

## Data availability

All sequencing data in this study has been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under accession numbers SUB15490056.

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