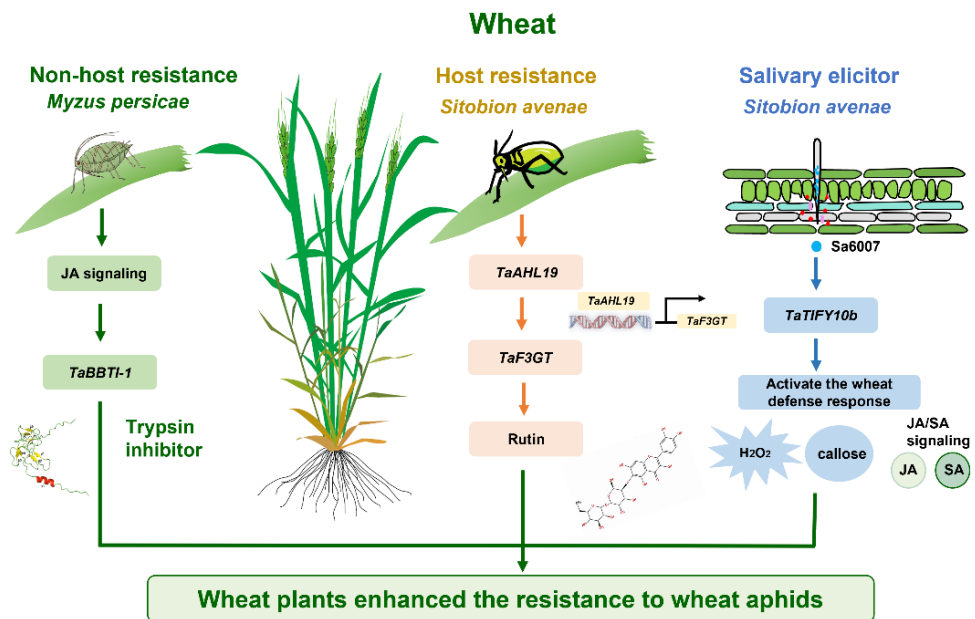


Aphid-resistant wheat and salivary proteins in plant defense responses



PhD Candidate: Xiaobei Liu

Promotors: Profs Frédéric Francis & Julian Chen

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UNIVERSITÉ DE LIÈGE – GEMBLoux AGRO-BIO TECH

Aphid-resistant wheat and salivary proteins in plant defense responses

Xiaobei Liu

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Abstract

Management of wheat aphids mainly relies on chemical insecticides, which has led to increasing concerns regarding pesticide residues and resistance, environmental pollution. Therefore, the development and cultivation of aphid-resistant wheat varieties are widely recognized as an effective- and sustainable- approach for aphid control and alternative measure to reduce over-reliance on chemical control. However, the poorly understanding of the mechanisms of wheat-aphid interactions has hindered aphid-resistant wheat breeding and application in agriculture. In this study, the mechanisms regulating wheat-aphid interactions were investigated from three aspects: 1) non-host resistance, 2) the aphid-resistant mechanisms of hexaploid wheat varieties, and 3) wheat immune response induced by aphid salivary proteins.

Firstly, using aphid bioassays and feeding behavior detection (electrical penetration graph, EPG), we used wheat seedling as a host for wheat aphid (*Sitobion avenae*), a poor-host for green peach aphid (*Myzus persicae*) and a non-host for pea aphid (*Acyrtosiphon pisum*), to establish a workflow for investigating aphid-induced poor-host resistant mechanisms. Compared with *S. avenae*, wheat plant pre-infested by *M. persicae* significantly suppressed the reproduction of aphid nymphs. Based on comparative transcriptomic analysis, *M. persicae* pre-infestation preferentially induced the expression of genes involved in α -linolenic acid metabolism, suggesting a prominent role for jasmonic acid (JA)-mediated defense responses during poor-host interactions. Also, *M. persicae* feeding, but not *S. avenae*, triggered a significant accumulation of endogenous JA in wheat plant. Furthermore, a Bowman-Birk trypsin inhibitor of wheat, designated as *TaBBTI-1*, was strongly induced by *M. persicae* infestation and was further upregulated by exogenous JA application. Moreover, artificial diets supplemented with recombinant TaBBTI-1 protein and overexpressing *TaBBTI-1* in wheat significantly impaired the fecundity of *S. avenae*.

Secondly, two hexaploid wheat (*Triticum aestivum*) cv. Hanmai 6172 (HM6172) and cv. Nongda 399 (ND399) were identified as aphid-resistant and aphid-susceptible wheat varieties, respectively. Age-stage life table and EPG analyses indicated that cv. HM6172 with antibiosis-resistance to aphids. Also, aphid infestation preferentially activated genes associated with phenylpropanoid and flavonoid (rutin and luteolin) biosynthesis while α -linolenic acid metabolism in cv. HM6172. Moreover, aphid feeding induced a significant accumulation of JA in cv. HM6172. In addition, dietary supplementation with rutin significantly reduced the fecundity of *S. avenae*, *Schizaphis graminum*, and *M. persicae*. Silencing *TaF3GT* (UDP-glucose: flavonol 3-O-glucosyltransferase), a key gene involved in rutin biosynthesis, via virus-induced gene silencing (VIGS) significantly reduced rutin accumulation in wheat. Furthermore, the AT-hook motif nuclear-localized protein 19 (*TaAHL19*) directly bound to the *TaF3GT* promoter and activated its expression, as demonstrated by yeast one-hybrid and dual-luciferase assays. Moreover, overexpression of *TaAHL19* significantly enhanced rutin accumulation and aphid resistance in wheat.

Thirdly, Sa6007, a salivary protein from *S. avenae* was identified as a candidate elicitor. Delivery of *Sa6007* into wheat leaves using a *Pseudomonas fluorescens*-

mediated bacterial type III secretion system induced hydrogen peroxide accumulation and callose deposition, indicating the activation of plant defense responses. Consistently, the overexpression of *Sa6007* in wheat (cv. Mingxian169) enhanced both salicylic acid (SA) and JA-mediated plant defense responses and significantly reduced aphid performance. Protein-protein interaction assays further showed that *Sa6007* directly interacts with the wheat transcription factor *TaTIFY10b* in plant nucleus.

In conclusion, this study elucidates key mechanisms underlying wheat resistance to aphids from three aspects, i.e. non-host resistance, host plant resistance, and salivary elicitor-triggered immunity. cv. HM6172 was identified as a valuable aphid-resistant wheat cultivar. TaBBTI-1 and the TaAHL19-TaF3GT-rutin modules provide promising genetic and metabolic resources for developing broad-spectrum wheat. From the aphid side, salivary elicitor *Sa6007* activates wheat defense and provides a potential target for manipulating induced resistance against aphids. Importantly, results from different parts of this thesis consistently support an important role for JA-associated defense responses in wheat resistance to aphids. These findings provide both mechanistic insights and practical genetic resources for the development of durable and sustainable aphid management strategies in wheat.

Keywords: *Sitobion avenae*, *Myzus persicae*, wheat-aphid interaction, plant defense response, salivary elicitor, antibiosis, rutin biosynthesis

Résumé

Le contrôle des pucerons dans les cultures de blé repose principalement sur l'utilisation d'insecticides chimiques, ce qui soulève des préoccupations croissantes concernant les résidus et l'émergence de populations résistantes aux insecticides ainsi que la pollution environnementale. Par conséquent, le développement de variétés de blé résistantes aux pucerons est largement reconnu comme une stratégie efficace et durable de lutte contre ces ravageurs. Toutefois, la compréhension encore limitée des mécanismes moléculaires régissant les interactions entre le blé et les pucerons constitue un obstacle majeur à la sélection et à l'exploitation de variétés résistantes. Dans cette étude, les mécanismes impliqués dans les interactions blé-puceron ont été étudiés sous trois angles: (i) la résistance de non-hôte; (ii) les mécanismes de résistance de variétés hexaploïdes de blé aux pucerons; et (iii) les réponses de défense du blé induites par les protéines salivaires aphidiennes.

À l'aide de bioessais, d'analyses du comportement alimentaire par électropénétrographie (EPG) et de profils transcriptomiques comparatifs, du blé a été utilisé comme plante hôte pour le puceron des céréales (*Sitobion avenae*), comme hôte médiocre pour le puceron vert du pêcher (*Myzus persicae*) et comme non-hôte pour le puceron du pois (*Acyrtosiphon pisum*). Une infestation préalable par *M. persicae* a significativement réduit la reproduction des générations ultérieures du puceron *S. avenae*. Cette pré-infestation a induit préférentiellement l'expression de gènes impliqués dans le métabolisme de l'acide α -linoléique, suggérant un rôle majeur des réponses de défense dépendantes de l'acide jasmonique (JA). Aussi, un inhibiteur de trypsine de type Bowman-Birk, désigné TaBBTI-1, a été fortement induit par l'infestation de *M. persicae* et davantage stimulé après traitement exogène au JA. L'ajout de la protéine recombinante TaBBTI-1 dans une diète artificielle ainsi que sa surexpression dans le blé ont significativement réduit la fécondité de *S. avenae*. De plus, l'expression de TaBBTI-1 chez le tabac (*Nicotiana tabacum*) a diminué de manière significative la sensibilité de la plante à *M. persicae*.

Concernant la résistance de la plante hôte, deux variétés hexaploïdes de blé (*Triticum aestivum*), Hanmai 6172 (cv. HM6172) et Nongda 399 (cv. ND399), ont été identifiées respectivement comme résistante et sensible aux pucerons. Les bioessais et les analyses EPG ont permis de conclure à une résistance de type antibiose pour le cv. HM6172. L'infestation par les pucerons a activé les voies métaboliques associées à la biosynthèse des phénylpropanoïdes, des flavonoïdes et au métabolisme de l'acide α -linoléique. En outre, une accumulation importante de JA a été observée après infestation. Plusieurs flavonoïdes, notamment la rutine et la lutéoline, se sont accumulés fortement chez cv. HM6172 après l'attaque des pucerons. L'incorporation de rutine dans une diète artificielle a réduit significativement la fécondité de *S. avenae*, *Schizaphis graminum* et *M. persicae*. Le silencage génique de TaF3GT (UDP-glucose: flavonol 3-O-glucosyltransférase), enzyme clé de la biosynthèse de la rutine, obtenu par la technique VIGS (Virus-Induced Gene Silencing), a entraîné une diminution significative de l'accumulation de rutine dans le blé. En outre, le facteur de transcription TaAHL19 (AT-hook motif nuclear-localized protein 19) s'est fixé

directement au promoteur de TaF3GT et active son expression, comme l'ont démontré les essais de levure un hybride (Y1H) et les essais de double luciférase. Sa surexpression dans le blé a considérablement augmenté l'accumulation de rutine ainsi que la résistance aux pucerons.

Enfin, la protéine salivaire Sa6007, issue de *S. avenae*, a été identifiée comme un éliciteur potentiel. Son introduction dans les feuilles de blé au moyen d'un système de sécrétion bactérien de type III médié par *Pseudomonas fluorescens* a induit une accumulation de peroxyde d'hydrogène ainsi qu'un dépôt de callose, témoignant de l'activation des réponses de défense végétales. De manière cohérente, la surexpression de Sa6007 dans le blé (cv. Mingxian169) a renforcé les voies de défense dépendantes de l'acide salicylique (SA) et du JA, tout en réduisant significativement les performances biologiques des pucerons. Les analyses d'interactions protéine-protéine ont montré que Sa6007 interagit directement avec le facteur de transcription du blé TaTIFY10b dans le noyau cellulaire.

En conclusion, cette étude a mis en évidence les principaux mécanismes de résistance du blé aux pucerons selon trois niveaux complémentaires : la résistance de non-hôte, la résistance de la plante hôte et la défense déclenchée par un éliciteur salivaire. La variété cv. HM6172 a été identifiée comme une ressource génétique particulièrement prometteuse pour la résistance aux pucerons. Les modules TaBBT1-1 et TaAHL19-TaF3GT-rutine constituent des ressources génétiques et métaboliques prometteuses pour le développement de blés à large spectre. Du côté des pucerons, l'éliciteur salivaire Sa6007 active les défenses du blé et représente une cible potentielle pour la manipulation de la résistance induite contre ces ravageurs. Il est important de noter que les résultats issus de différentes parties de cette thèse soutiennent de manière cohérente le rôle important des réponses de défense associées à l'acide jasmonique dans la résistance du blé aux pucerons. Ces travaux fournissent à la fois de nouvelles connaissances fondamentales sur les interactions blé-puceron et des ressources génétiques utiles au développement de stratégies durables de protection du blé contre les pucerons.

Mots-clés: *Sitobion avenae*, *Myzus persicae*, interaction puceron du blé, réponse de défense des plantes, éliciteur salivaire, antibiose, biosynthèse de la rutine

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List of acronyms

3G6RhaT	Flavonol 3-O-glucoside rhamnosyltransferase
ABA	Abscisic acid
AbaA	Aureobasidin A
BiFC	Bimolecular fluorescence complementation
BSMV	Barley stripe mosaic virus
Bxs	Benzoxazinoids
BYDV	Barley yellow dwarf virus
CIMMYT	International Maize and Wheat Improvement Center
CRISPR/Cas9	Clustered Regularly Interspaced Palindromic Repeats/CRISPR-associated protein 9
Co-IP	Co-immunoprecipitation
DAB	3'-Diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DCXR	Diacetyl/L-xylulose reductase
DEGs	Differentially expressed genes
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one
DMNT	(E)-4,8-dimethyl-1,3,7-nonatriene
DMSO	Dimethyl sulfoxide
dsRNA	double-stranded RNA
EBF	(E)- β -farnesene
EPG	Electrical penetration graph
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FDR	False discovery rates
f_{xj}	Age-specific female fecundity
GBS	Genotyping by sequencing
GO	Gene ontology
GWAS	Genome-wide association study
H ₂ O ₂	Hydrogen peroxide
HAMPs	Herbivore-associated molecular patterns
HIPVs	Herbivore-induced plant volatiles
HM6172	Hanmai 6172
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IPTG	isopropyl- β -D-thiogalactopyranoside
JA	Jasmonic acid
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LIC	Ligation-independent cloning
LUC	Dual- Luciferase
l_x	Age-specific survival rate
MAPK	Mitogen-activated protein kinase
MeSA	Methyl salicylate

MHO	6-Methyl-5-hepten-2-one
MRM	Multiple reaction monitoring
m_x	Age-specific fecundity
ND399	Nongda 399
NHR	Non-host resistance
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PIs	Protease inhibitors
PR1	Pathogenesis-related protein 1
PRRs	Pattern-recognition receptors
PTI	PAMP-triggered immunity
QTL	Quantitative trait locus
R_0	Net reproductive rate
REN	Renilla luciferase
RILs	Recombinant inbred lines
r_m	Intrinsic rate of increase
RNAi	RNA interference
RNA-Seq	Transcriptome sequencing
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative PCR
SA	Salicylic acid
SaCDK	Cyclin-dependent kinase-like gene of <i>S. avenae</i>
SBBI	Soybean Bowman-Birk inhibitor
SD	Synthetic defined
SNPs	Single-nucleotide polymorphisms
SPc	Star polycation
s_{xj}	Age-stage-specific survival rate
T	Mean generation time
T3SS	Type III secretion system
Ta4CL	4-Coumarate-CoA ligase
TaACX	Acyl coenzyme A oxidase
TaACX1	Acyl-CoA oxidase 1
TaAHL19	AT-hook motif nuclear-localized protein 19
TaAOC	Allene oxide cyclase
TaAOS	Allene oxide synthase
TaBBTI-1	Bowman-Birk trypsin inhibitors
TaC4H	Cinnamic acid 4-hydroxylase
TaCAD	Cinnamyl-alcohol dehydrogenase
TaCBSX3	CBS domain-containing protein CBSX3
TaCCR	Cinnamoyl-CoA reductase
TaCHI	Chalcone isomerase
TaCHS	Chalcone synthase
TaE22	Ubiquitin-conjugating enzyme E22
TaF3'H	Flavonol 3'-hydroxylase

TaF3GT	UDP-glucose: flavonol 3-O-glucosyltransferase
TaF3H	Flavonol 3-hydroxylase
TaFAD	Fatty acid desaturase
TaFAH	Ferulate 5-hydroxylase
TaG6P1E	Glucose-6-phosphate 1-epimerase
TaJMT	Jasmonic acid carboxyl methyltransferase
TaLOX	Lipoxygenase
TaMFP	Multifunctional protein
TaOMT	O-methyltransferase
TaOPCL1	OPC-8: 0 CoA ligase 1
TaOPR	12-Oxo-phytodienoic acid reductase
TaPAL	Phenylalanine ammonia-lyase
TaPHOS34	Universal stress protein PHOS34
TaPKT3	Peroxisomal 3-ketoacyl-CoA thiolase 3
TaTMTT	Trimethyltridecatetrane synthase
UGTs	UDP-dependent glucosyltransferases
VIGS	Virus-induced gene silencing
VOCs	Volatile organic compounds
VSP1	Vegetative storage protein1
WYMV	Wheat yellow mosaic virus
X- α -Gal	5-bromo-4-chloro-3-indoxyl- α -d-galactopyranoside
Y1H	Yeast One-Hybrid
λ	Finite rate of increase

Chapter 1

Plant-aphid interactions in cereal crops: wheat plant defense and aphid saliva- mediated coevolutionary arm race

Adapted from:

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Abstract

Wheat aphids are among the most destructive pests of wheat, posing a serious threat to wheat production and global food security. The development of aphid-resistant wheat varieties is widely regarded as one of the most economical and sustainable strategies for aphid management. A comprehensive understanding of the plant defense mechanisms is fundamental to the breeding of resistant wheat germplasm. Recent studies have revealed that aphid salivary proteins play critical roles in manipulating host defenses, whereas wheat has evolved multiple layers of defense to perceive and respond to aphid attack. In this review, we summarize current advances in wheat-aphid interactions, focusing on the roles of aphid salivary effectors, aphid-induced immune signaling, physical and chemical defense barriers, herbivore-induced plant volatiles (HIPVs), resistance genes, and non-host resistance mechanisms. By integrating aphid effector biology with host immunity and resistance mechanisms, this review provides a conceptual framework for understanding wheat-aphid interactions and for developing durable and environmentally sustainable resistance strategies.

Key words: wheat aphids; plant defense; non-host resistance; resistance genes; salivary proteins

1. Introduction

Wheat is an important crop widely cultivated around the world and plays a critical role in global food security (Zhang et al., 2022b). Wheat aphids are among the most destructive pests of wheat, as they feed directly on phloem sap (Blackman and Eastop, 2006). Characterized by their small body size, short generation time, high reproductive capacity, and rapid population growth, they pose a serious threat to wheat production throughout all stages of crop development (Blackman and Eastop, 2006). The main species of wheat aphids include *Sitobion avenae*, *Rhopalosiphum padi*, *Schizaphis graminum*, *Metopolophium dirhodum*, and *Diuraphis noxia*. Currently, chemical pesticides remain heavily relied upon for the management of wheat aphids, leading to a series of problems including pest resistance, adverse effects on beneficial insects and environmental pollution (Bass and Jones, 2018). Therefore, it is urgent to reduce the use of chemical pesticides and enhance the ecological benefits of wheat-based agroecosystems. Breeding and utilizing aphid-resistant wheat varieties is one of the most cost-effective strategies for sustainable aphid management. However, there is a lack of aphid-resistant germplasm among existing wheat varieties.

In the “arms race” of coevolution between plants and herbivorous insects, plants have evolved diverse direct and indirect defense mechanisms to counter insect feeding, thereby enhancing plant resistance to insect pests (Mithöfer and Boland, 2012). During plant defense responses, signaling molecules such as phytohormones play crucial roles in regulating defense signaling networks (Erb and Kliebenstein, 2020). Plant resistance can be categorized into non-host and host resistance, of which the latter has been more extensively studied. Host resistance to aphids is generally exhibited through antixenosis, antibiosis, and tolerance, which are mediated by a combination of constitutive and inducible defense mechanisms (Painter, 1951).

Considerable progress has been made in understanding wheat resistance to aphids, particularly in the areas of plant defense signaling, resistance genes, and aphid-induced defense responses. However, the molecular mechanisms governing wheat-aphid interactions remain incompletely understood. In recent years, aphid salivary proteins have emerged as key determinants of plant-aphid interactions, functioning either as elicitors that activate host defenses or as effectors that suppress plant immunity (Hogenhout and Bos, 2011; Snoeck et al., 2022). However, current identification of aphid salivary effectors and elicitors is limited to functional studies of individual salivary proteins. Their molecular targets in wheat have not been explored in depth, and the signaling network connecting aphid perception and effective resistance response has not been fully elucidated. In addition, only a limited number of aphid-resistance genes have been characterized and utilized in breeding programs, which has constrained the development of wheat cultivars with durable and broad-spectrum resistance. Meanwhile, non-host resistance, which is often more durable and broad-spectrum than host resistance (Mysore and Ryu, 2004; Senthil-Kumar and Mysore, 2013), remains relatively underexplored in wheat-aphid systems despite its considerable potential for resistance breeding.

To address these challenges, this review summarizes current knowledge of wheat-aphid interactions from the perspective of the molecular arms race between aphids and their host plants. We focus on the roles of aphid salivary proteins in modulating plant defenses and discuss the multiple layers of wheat resistance, including defense signaling pathways, physical and chemical barriers, herbivore-induced plant volatiles, resistance genes, and non-host resistance mechanisms. This review aims to provide new insights for further research on aphid resistance mechanisms and the genetic basis of resistance in wheat, ultimately supporting efficient and sustainable crop protection.

2. Plant immune perception and anti-defense strategies mediated by aphid saliva

Aphids are piercing-sucking insects that penetrate the sieve elements of the phloem with their stylets to ingest plant sap, acquiring sugars, amino acids, and other nutrients. During probing and feeding, aphids secrete two types of saliva: gelling saliva and watery saliva. The gelling saliva hardens to form a sheath around the stylets with a protective function. Watery saliva contains complex proteins, including detoxification enzymes, hydrolases, proteases, calcium-binding proteins, and salivary proteins.

There is a complex dynamic interaction between pathogens and host plants, and the “zig-zag model” provides a conceptual framework for illustrating this relationship (Jones and Dangl, 2006). Some conserved molecules in aphid saliva are similar to pathogen-associated molecular patterns (PAMPs) and are perceived by pattern-recognition receptors (PRRs) on the plant cell surface, thereby triggering PAMP-triggered immunity (PTI). Aphid then secretes effector proteins into the host plant to interfere with PTI, resulting in effector-triggered susceptibility (ETS). However, some plants can evolve a resistance gene (R gene) encoding proteins that recognize these effectors and trigger effector-triggered immunity (ETI), further preventing aphid infestation. Aphids can regulate plant defense responses by secreting salivary proteins into the plant through the stylets. These salivary proteins can be classified into two categories: elicitors, which activate plant defense responses, and effectors, which suppress plant defense (Hogenhout and Bos, 2011; Snoeck et al., 2022). Recent advances in the study of the transcriptomes and proteomes of salivary glands have greatly expanded the identification of candidate salivary proteins in wheat aphids, providing an essential foundation for functional analysis of salivary proteins (Zhang et al., 2017b; 2021). Integrative transcriptomic and proteomic analyses have identified 76 and 114 salivary proteins from the watery saliva of *S. avenae* and *S. graminum*, respectively (Zhang et al., 2021). Recently, RNA interference (RNAi) has emerged as a promising strategy for silencing target genes and holds great potential for the research of salivary proteins (Niu et al., 2021; Yan et al., 2020). The use of double-stranded RNA (dsRNA) to specifically silence key genes essential for pest growth and development not only facilitates pest control but also minimizes risks to non-target organisms, particularly the natural enemies of pests. This will facilitate the discovery of key virulence effectors and salivary elicitors, which are of great significance for the breeding and utilization of aphid-resistant wheat varieties.

2.1. Wheat defense responses induced by salivary elicitors

Plants possess the ability to rapidly and specifically perceive herbivore-derived cues, such as herbivore-associated molecular patterns (HAMPs) and salivary elicitors, which play a pivotal role in activating plant defense responses against herbivore attack (Acevedo et al., 2015). Recognition of these signals typically initiates PTI, leading to a cascade of downstream defense. A previous study found that treatment of *A. thaliana* with 3-10 kDa salivary protein fragments from *M. persicae* significantly upregulated the expression of defense-related genes such as *PAD3*, *PR2*, and *WKRY28*, while simultaneously leading to a significant decrease in aphid fecundity (Vos and Jander, 2009). Similarly, the symbiont-derived protein GroEL from *M. euphorbiae* was shown to induce PTI response in Arabidopsis, including callose deposition and ROS accumulation, ultimately suppressing aphid performance (Chaudhary et al., 2014). The salivary protein Me47 from *M. euphorbiae* induced the expression of PTI-associated genes, including *Lrr22*, *Pti5*, *Gras2*, and *PR1a*, in tomato plants (Kettles and Kaloshian, 2016). Moreover, the salivary protein Cathepsin B from *M. persicae* interacts with the phloem-associated protein EDR1, resulting in sustained activation of ROS signaling in vascular tissues. This interaction interferes with aphid feeding behavior and reduces host suitability (Guo et al., 2020). Collectively, these findings highlight that aphid salivary elicitors are key modulators of plant immune signaling.

Advances in functional genomics have facilitated the identification and characterization of aphid salivary effectors in cereal systems. For instance, the Type III secretion system (T3SS) of *Pseudomonas fluorescens* EtAnH has been successfully employed to deliver candidate effectors into wheat leaves for transient overexpression, providing a powerful tool for functional validation (Bai et al., 2021). In wheat systems, the identification and functional characterization of aphid salivary elicitors are essential for elucidating plant defense mechanisms and facilitating the discovery of resistance-related genes. Infiltration of watery saliva from *S. avenae* into wheat leaves significantly upregulated the expression of SA-related genes, such as *PAL* and *PR1*, thereby negatively affecting aphid feeding behavior and development. These results demonstrate that aphid salivary proteins can directly participate in the induction of wheat defense responses (Zhang et al., 2017a). To date, only two salivary protein elicitors of *S. avenae* have been identified (Table 1-1). Among them, the small molecular protein GroES, derived from the primary endosymbiont *Buchnera aphidicola* of *S. avenae*, can induce ROS accumulation and callose deposition in wheat, thereby enhancing the resistance of wheat to aphids (Li et al., 2022). In addition, the salivary protein SmCSP4 from *S. avenae* interacts with the wheat transcription factor *TaWRKY76*, activating SA-mediated defense responses and further strengthening host resistance (Zhang et al., 2023).

2.2. Salivary effector-mediated anti-defense strategies of wheat aphids

During evolution, aphid salivary effectors have acquired critical roles in modulating aphid-plant interactions by suppressing host defense responses and thereby enhancing

aphid adaptability. Early functional characterization of aphid salivary effectors was conducted in the pea aphid *A. pisum* (Mutti et al., 2006). RNAi-mediated silencing of the salivary gland-specific gene *C002* in *A. pisum* impaired phloem feeding and significantly reduced aphid survival and fecundity, demonstrating that *C002* is essential for host colonization (Mutti et al., 2008). Subsequent studies further demonstrated that multiple aphid salivary proteins function as effectors that suppress plant immunity. Transient overexpression of *M. persicae* salivary proteins MpC002, Mp1/PIntO1, and PintO2, as well as *M. euphorbiae* salivary proteins Me10 and Me23, in tobacco and Arabidopsis significantly increased aphid reproduction, suggesting important roles in suppressing host defense responses (Bos et al., 2010; Atamian et al., 2013). Similarly, overexpression of salivary protein Mp55 in *A. thaliana* enhanced aphid performance and host preference, whereas silencing *Mp55* markedly reduced aphid reproduction, confirming its function as a key effector promoting infestation (Elzinga et al., 2014). In addition to directly modulating plant immunity, some aphid effectors function through metabolic interference. For example, the salivary protein diacetyl/L-xylulose reductase (DCXR) from *Aphis craccivora* participates in methylglyoxal detoxification, and its transient expression in pea significantly increased aphid fecundity (MacWilliams et al., 2020). Furthermore, previous studies have shown that other important piercing-sucking pests, such as *B. tabaci* and the *Nilaparvata lugens*, also secrete salivary effectors that suppress host plant defense responses (Huang et al., 2023; Xu et al., 2019), suggesting that effector-mediated immune suppression represents a conserved strategy among phloem-feeding insects.

Several salivary effectors have subsequently been functionally characterized in wheat aphids (Table 1-1). The salivary protein Rp1 from *R. padi* suppresses defense responses in barley, thereby facilitating aphid colonization (Escudero-Martinez et al., 2020). Likewise, transient expression of the salivary protein Sg2204 from *S. graminum* significantly reduced callose deposition in wheat leaves (Zhang et al., 2022d). Two salivary effectors from *S. avenae*, Sm10 and SmC002, enhance host plant susceptibility and promote aphid growth by suppressing JA-mediated defense responses (Fu et al., 2023). In addition, the salivary effector Sm9723 suppresses the expression of defense-related genes associated with both JA and SA signaling pathways, enabling aphids to evade plant immunity (Zhang et al., 2022e).

Table 1-1: Salivary elicitors and effectors of wheat aphids.

Salivary protein	Aphid species	Functions	References
SmCSP4	<i>S. avenae</i>	Activate SA defense pathway	Zhang et al., 2023
GroES	<i>S. avenae</i>	Trigger defense responses	Li et al., 2022
Sm10	<i>S. avenae</i>	Enhance host plant susceptibility	Fu et al., 2023
SmC002	<i>S. avenae</i>	Enhance host plant susceptibility	Fu et al., 2023
Sm9723	<i>S. avenae</i>	Suppress plant immunity	Zhang et al., 2022e
SaCDK	<i>S. avenae</i>	Inhibit host defense response	Zhang et al., 2024
SaE23	<i>S. avenae</i>	Suppress wheat plant defenses	Shang et al., 2025
SaApo AI	<i>S. avenae</i>	Inhibit plant hypersensitive response	Zheng et al., 2026

Sg2204	<i>S. graminum</i>	Suppress wheat defense	Zhang et al., 2022d
RP1	<i>R. padi</i>	Suppress barley defense responses	Escudero-Martinez et al., 2020

Figure 1-1 illustrates how salivary proteins manipulate the immune regulation of plants. Compared with salivary elicitors, salivary effectors are secreted by aphids to suppress or evade host immunity, thereby facilitating successful feeding and reproduction. This represents an evolutionary anti-defense mechanism developed by aphid against plants. Moreover, the outcome of plant-aphid interactions is determined not by individual salivary proteins but by the combined action of complex salivary proteins mixtures that integrate both defense and anti-defense functions. A deeper understanding of the balance and interplay between elicitors and effectors will be essential for elucidating the molecular mechanisms underlying aphid adaptation and for developing durable strategies for crop resistance.

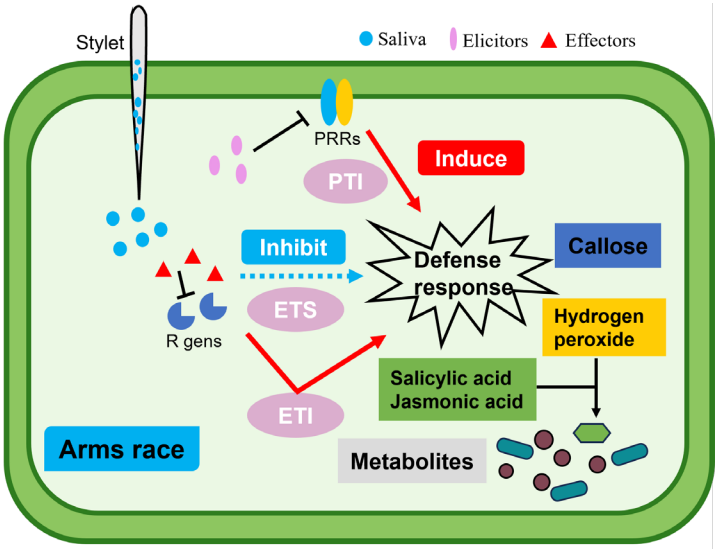


Figure 1-1: A model depicting the manipulation of plant immunity by aphid salivary protein.

At present, functional studies on salivary proteins in wheat aphids remain at an early stage, particularly in *S. avenae*, *S. graminum*, and *R. padi*. Notably, these three major wheat aphid species exhibit distinct feeding preferences and damage characteristics on wheat, suggesting that they may employ different salivary effector strategies during infestation. Although several salivary proteins with potential roles in suppressing host immunity, inducing plant defense response, or facilitating phloem feeding have been identified, most studies remain limited to the functional characterization of individual candidate proteins. A systematic understanding of aphid effector repertoires, effector cooperation, and species-specific virulence strategies among wheat aphids is still lacking. Consequently, research on salivary effector-

mediated aphid virulence and host adaptation remains far less advanced than that in other plant-insect systems.

3. Early defense signaling in wheat under aphid infestation

Plant hormones play an important role in the plant immune response (Erb and Kliebenstein, 2020). In response to insect infestation, wheat can trigger plant defense pathways mediated by phytohormones such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA), as well as signaling molecules like reactive oxygen species (ROS). These pathways subsequently induce the accumulation of secondary metabolites, thereby affecting aphid growth and development (Hu and Zhao, 2014). Hydrogen peroxide (H_2O_2), as a broad-spectrum signaling molecule, accumulates within plant tissues in response to both pest and pathogen infestation (Choudhury et al., 2013). Feeding by *S. avenae* and *S. graminum* leads to a significant increase in H_2O_2 content in wheat leaves, with *S. graminum* inducing a stronger accumulation of H_2O_2 (Zhang et al., 2019b).

The JA and SA pathways are two crucial hormone signaling pathways associated with plant defense to insect. Changes in defensive metabolite levels mediated by the interaction between these pathways further influence plant resistance. The SA pathway is reported to primarily mediate plant defense against aphids. For instance, feeding by *S. avenae* induces elevated the expression of *pathogenesis-related protein 1* (*PR1*) in the SA pathway of wheat (Zutter et al., 2017). Transcriptome sequencing (RNA-Seq) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses have also revealed that the infestation by both *S. avenae* and *S. graminum* induces an SA-mediated defense response in wheat, leading to increased SA content (Zhang et al., 2019a). Previous studies have shown that wheat aphid infestation also induces the expression levels of JA-related genes and increases JA content (Liu et al., 2020; Reddy et al., 2013). Spraying wheat seedlings with JA induces an increase in the content of defensive compound in wheat, such as DIMBOA, thereby significantly inhibiting the development and fecundity of wheat aphids (Yin et al., 2005). Although previous studies have indicated that JA and SA are antagonistic, increasing evidence suggests that JA and SA can also act synergistically or independently, depending on factors such as plant species (Thaler et al., 2012; Moreira et al., 2018; Jiao et al., 2022). Additionally, ET enhance plant defense mechanisms against aphids by promoting cell wall thickening (Rasool et al., 2017). Feeding by wheat aphids can induce upregulation of the ET pathway marker gene *ETN2* in harpin transgenic wheat lines and inhibit the performance of wheat aphids (Xu et al., 2014).

4. Multi-layered wheat defense strategies against aphids

The perception of aphid attack and the activation of downstream signaling pathways ultimately lead to the deployment of diverse defense responses in wheat. These defense responses represent the functional outputs of plant immunity and play crucial

roles in limiting aphid colonization, feeding, and population growth. Following aphid recognition, wheat activates a multilayered defense system that includes structural barriers, the production of defensive metabolites, and indirect defenses mediated by herbivore-induced plant volatiles (HIPVs). Physical defense traits that contribute to resistance against aphids include morphological characteristics, epicuticular waxes, trichomes, and internal structures. These physical barriers constitute the first line of plant defense, interfering with aphid host selection, feeding behavior, growth, and development. Similarly, chemical defense plays a critical role in the plant defense system, involving secondary metabolites, nutrient composition, and other biochemical factors that adversely affect aphid physiology and survival.

4.1. Morphological and physical defense against aphids in wheat plants

When aphids migrate into wheat fields in spring, they initially locate host plants by employing olfactory and visual cues, such as plant color and volatile organic compounds released by the wheat (Dorling, 2014). The color of wheat plants serves as a visual cue influencing aphid host selection. When aphids select plants and land on their surfaces, tactile cues serve as the basis for assessing host plant suitability for colonization. Morphological traits of wheat, such as leaf trichomes, flag leaf characteristics, awn length, and leaf surface wax, constitute important factors influencing aphid colonization. The length and density of leaf trichomes are significantly positively correlated with wheat resistance levels against aphid (Wang et al., 2014). The density of leaf trichomes constitutes a physical barrier to aphid feeding, serving as a key determinant of wheat defense. For example, aphids prefer to feed on older leaves, which have lower trichome density (Singh et al., 2021). Plant surface wax can significantly affect the selection, feeding, and oviposition of aphids. For instance, the wax on wheat leaves inhibits the aphid feeding behavior and exhibits toxic effects on aphids (Wojcicka, 2016). Callose is a β -1,3-linked glucan that is typically deposited in small amounts on the surface or around the sieve pores of phloem sieve plates, and its deposition is associated with plant defense to pests and pathogens (Nalam et al., 2019; Malinovsky et al., 2014). Feeding by *S. avenae* and *S. graminum* induce substantial callose accumulation in wheat leaf cells (Choudhury et al., 2013). However, the precise mechanism of callose involvement in wheat-aphid interactions remain unclear. It is hypothesized that aphid-induced callose deposition may obstruct the transport of phloem sap to aphid stylets, thereby inhibiting aphid feeding and enhancing wheat resistance.

4.2. Chemical defense against aphid in wheat plant

4.2.1. Nutritional components

The nutrients that aphids ingest from wheat leaves affect their growth performance after aphids settle on the wheat. Soluble proteins, sugars, amino acids, and inorganic salts in wheat directly impact aphid growth and development, thereby contributing to plant defense against aphid infestation (Yang et al., 2024). A previous study showed that the total protein content in the leaves of aphid-resistant wheat varieties was

significantly lower than that in aphid-susceptible varieties, whereas the content of free amino acids in the phloem was higher than that in susceptible varieties (Liu et al., 2017). Under high temperature and drought stress, wheat plants exhibit increased soluble sugar and amino acid content and aphids exhibit lower net reproductive rates, intrinsic rates of increase, and finite rates of increase (Xie et al., 2020). Both the diversity and abundance of amino acids in wheat are associated with wheat resistance to aphids. β -aminobutyric acid has been shown to provide an inhibitory effect on wheat aphids. For example, root irrigation with β -aminobutyric acid during the wheat seedling stage significantly inhibited the weight gain of wheat aphids. Furthermore, adding β -aminobutyric acid to artificial diet resulted in a 55% reduction in aphid body weight, indicating a direct toxic effect on aphids (Cao et al., 2014). However, the relationship between different amino acids and wheat resistance levels to aphids is not consistent. For instance, the contents of glutamic acid, alanine, lysine, and aspartic acid in wheat leaves are positively correlated with the intrinsic growth rate of the wheat aphids, whereas the contents of leucine, isoleucine, valine, and proline are negatively correlated with aphid intrinsic growth rates (Gao et al., 1998).

4.2.2. Secondary metabolites

Plant secondary metabolites are products of complex branched metabolic pathways, and most of these compounds enhance resistance to pests and diseases (Erb and Kliebenstein, 2020). Most secondary metabolites function as phytoalexins that enhance plant resistance to pests and diseases (Nalam et al., 2019). The secondary metabolites associated with wheat plant defense mainly include phenols, benzoxazinoids (Bxs), and alkaloids.

Among plant secondary metabolites, phenolic compounds constitute one of the most common and widespread groups of defense-related chemicals (Yang et al., 2024). Previous studies indicate that the levels of plant phenols are positively correlated with plant resistance to insects, as these compounds exert detrimental effects on aphid feeding behavior, growth, and development (Tlak and Dar, 2021). The biosynthesis of phenolic compounds is largely regulated by the phenylpropanoid pathway, which plays a crucial role in plant defense. For instance, phenylpropanoid pathway has been shown to contribute to wheat kernel resistance against *Sitodiplosis mosellana* infestation (Wang et al., 2022b). Lignin, a phenolic heteropolymer, confers resistance to herbivores by both creating a mechanical barrier through increased leaf toughness and reducing the nutritional content of leaves (Johnson et al., 2009). The content of *p*-coumaric acid and vanillic acid in wheat seedling leaves was significantly and positively correlated with the resistance level against aphids (Gebretsadik et al., 2022). Flavonoids are a major branch of phenolic compounds and play an important role in mediating plant responses to biotic and abiotic stresses (Shen et al., 2022). As cytotoxic secondary metabolites, flavonoids protect plants from insect damage by influencing the behavior and development of insects (Simmonds, 2003). For example, the flavone tricetin in wheat exerted strong antifeedant effects against *S. graminum* (Dreyer and Jones, 1981). Similarly, supplementation of artificial diets with catechins and quercetin significantly reduced the survival rate of *S. avenae* and *R. padi*, and also adversely affected their growth and development (Chen et al., 2002). Consistent with

these findings, high-performance liquid chromatography (HPLC) analysis has revealed a significant positive correlation between quercetin accumulation in wheat kernels and flag leaves and plant resistance to aphids (Hu and Zhao, 2014).

Bxs represent an important class of secondary metabolites that play a crucial role in chemical defense against a wide range of biotic stresses (Zhou et al., 2018). Integrative transcriptomic and metabolomic analyses have demonstrated that Bxs function as direct defensive compounds against *Ostrinia furnacalis* in maize (Guo et al., 2019). In wheat, Bxs were present in higher concentrations in older wheat leaves than in younger leaves, and that these compounds were a key factor in determining wheat resistance levels to aphids (Singh et al., 2021). Among Bxs, hydroxamic acids are considered the most biologically active group (Niemeyer, 2009). The compound 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is the predominant hydroxamic acid derivative in wheat. Elevated DIMBOA levels are positively correlated with enhanced wheat resistance to *R. padi* and *S. avenae* (Yan, 1995). It functions as a digestive toxin by modulating trypsin activity in *Ostrinia nubilalis* larvae (Houseman et al., 1992). In addition, both DIMBOA and its precursors have been shown to reduce UDP-glucuronosyltransferase activity in *S. avenae* (Leszczynski et al., 1992). Furthermore, the content of Bxs compounds in wheat is altered by aphid feeding, thereby affecting callose deposition and consequently influencing the plant resistance to aphids.

4.2.3. Herbivore-induced plant volatiles (HIPVs)

Herbivore attack triggers a wide array of plant defense responses, among which HIPVs represent one of the most rapid and ecologically responses (War et al., 2011). Volatile compounds released by host plants serve as crucial cues for aphids in locating suitable hosts for settlement. In response to aphid infestation, plants emit complex blends of volatile organic compounds (VOCs), particularly terpenoids, including (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)- β -farnesene (EBF), and linalool. HIPVs mediate tritrophic interactions by simultaneously influencing herbivores and their natural enemies. For example, EBF serves both as an alarm hormone for aphids and as a signaling molecule for plants. It functions to repel aphids while simultaneously attracting predatory and parasitic natural enemies, thereby playing an important role in mediating plant-aphid interactions (Jiang et al., 2023). Aphid infestation has been shown to induce the release of several VOCs, including 2-camphene, 6-methyl-5-hepten-2-one (MHO), and methyl salicylate (MeSA), along with elevated emissions of S-linalool, trans-2-hexenal, and benzaldehyde. Behavioral assays have demonstrated that MHO and MeSA emitted by aphid-resistant wheat varieties exert a significant repellent effect on wheat aphids (Li et al., 2017). Similarly, the monoterpene linalool released by octoploid dwarf wheat exerted a repellent effect on wheat aphids (Zhan et al., 2021). Importantly, field applications of HIPVs such as MeSA and EBF have proven effective in simultaneously repelling aphids and attracting natural enemies, thereby reducing aphid damage in wheat agroecosystems (Dong et al., 2012).

Taken together, wheat's defense against aphids is not mediated by a single mechanism but rather by the coordinated activation of multiple defense layers. Following aphid attack, defense signaling pathways trigger a series of downstream responses, including physical and chemical defenses and HIPV-mediated indirect defenses (Figure 1-2). These responses reshape aphid host selection, feeding behavior, survival, and fecundity, thereby contributing to wheat resistance. Importantly, the activation of these defense responses is under genetic control, suggesting that resistance genes play a central role in shaping resistance outcomes.

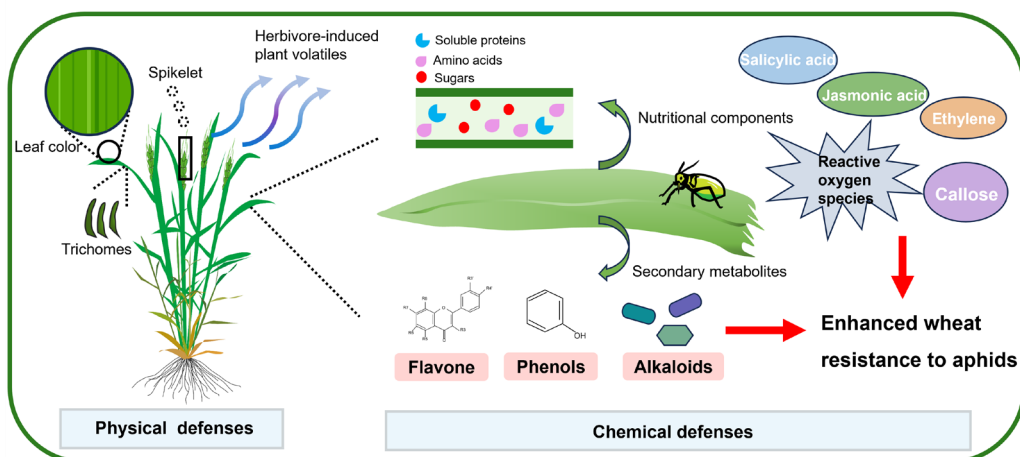


Figure 1-2: Physical and chemical defenses against aphids in wheat.

5. Genetic basis of wheat resistance

Although the defense mechanisms described above are phenotypic manifestations of resistance, their effectiveness is ultimately determined by underlying genetic factors. Resistance genes represent a direct form of plant defense to herbivorous insects (Radchenko et al., 2022). Molecular breeding for crop resistance to pests and pathogens is one of the most economical and effective approaches for efficient control. Molecular mapping of aphid resistance genes in crops enables the selection of resistant traits, significantly shortening the breeding process for aphid-resistant wheat varieties (Wang et al., 2015). The complex genetic background of wheat, coupled with its allopolyploid nature, confers high tolerance to genomic modifications. Previous studies have demonstrated that genes conferring resistance to pests, pathogens and abiotic stresses can be successfully introduced into wheat from other cereal crops, including rye, barley and oats (Radchenko et al., 2022).

Dn1 was identified as the first resistance gene against *D. noxia* in wheat. To date, more than 10 resistance genes against *D. noxia* and 15 resistance genes against *S. graminum* have been identified in wheat (Table 1-2) (Radchenko et al., 2022; Toit, 1987). Currently, the identified resistance genes to *S. avenae* are mainly located on wheat chromosomes 6A and 7D (Table 1-2). For example, the resistance gene *RA-1*

against *S. avenae*, derived from the durum wheat accession C273, was localized to chromosome 6AL through linkage mapping (Liu et al., 2012). Furthermore, the resistance gene *Sa2*, identified in the Chinese wheat accession XN98-10-35, is located on chromosome 7D (Wang et al., 2015). In addition, through combined analysis of aphid performance and wheat genotypes, broad-spectrum resistance genes against *S. avenae*, *R. padi*, and *S. graminum* were identified in the wheat lines E12165, Amigo, and *Presto triticale* and were mapped to chromosome 1AL.1RSam (Crespo-Herrera et al., 2013). By reducing genome complexity using restriction endonucleases, genotyping by sequencing (GBS) offers an efficient platform for gene mapping in wheat. Researchers at the International Maize and Wheat Improvement Center (CIMMYT) used GBS to screen a recombinant inbred lines (RILs) population derived from the aphid-resistant wheat accession CWI76364 and the susceptible accession Seri M82. The analysis identified a quantitative trait locus (QTL) conferring antibiotic resistant to *R. padi* (*QRp.slu.4BL*) on wheat chromosome 4BL, as well as two QTLs associated with tolerance to *R. padi* (*QRp.slu.5AL* and *QRp.slu.5BL*) on chromosomes 5AL and 5BL, respectively (Crespo-Herrera et al., 2014). In addition, evaluation of an F₆ RILs population (n=226) derived from a cross between *S. graminum*-resistant line Sokoll and the susceptible line Weebill1 led to the identification of a major resistance locus on chromosome 7DL. Subsequent linkage and QTL analyses enabled preliminary mapping of the resistance gene, designated *GbSk1* (Crespo-Herrera et al., 2019).

Table 1-2: Wheat aphid resistance genes.

Resistance genes	Chromosome	Aphid species	Crops	References
<i>RA-1</i>	6AL	<i>S. avenae</i>	<i>T. durum</i>	Liu et al., 2012
<i>Sa2</i>	7D	<i>S. avenae</i>	<i>T. aestivum</i>	Wang et al., 2015
<i>Gb1</i>	-	<i>S. graminum</i>	<i>T. aestivum</i>	Tyler et al., 1987
<i>Gb2, Gb6</i>	1A	<i>S. graminum</i>	<i>S. cereale</i>	Hollenhorst et al., 1983; Porter et al., 1994
<i>Gb5</i>	7AL	<i>S. graminum</i>	<i>Ae. speltoides</i>	Dubcovsky et al., 1998
<i>Gb3, Gb4, Gb7, Gb8, Gb9, Gba, Gbb, Gbc, Gbd, Gbx1, Gbz</i>	7DL	<i>S. graminum</i>	<i>Ae. tauschii</i>	Weng and Lazar, 2002; Weng et al., 2005; Zhu et al., 2004; 2005; Xu et al., 2020; 2024
<i>SgR1</i>	-	<i>S. graminum</i>	<i>S. bicolor</i>	Zhang et al., 2025
<i>Dn1, Dn2, Dn5, Dn6, Dn8, Dnx, Dn2401, Dn626580, Dn100695</i>	7DS	<i>D. noxia</i>	<i>Ae. tauschii</i>	Toit, 1987; Saidi et al., 1996; Liu et al., 2001; Harvey et al., 1990; Fazel-Najafabadi et al., 2015; Valdez et al., 2012; Aykut et al., 2016
<i>Dn7</i>	1B	<i>D. noxia</i>	<i>S. cereale</i>	Marais et al., 1994

Although many resistance genes have been identified, the underlying molecular mechanisms remain unclear. The completion of the wheat whole-genome sequencing, together with the emergence of genome-wide association study (GWAS) approaches, has greatly advanced functional genomics research in wheat (Sehgal and Dreisigacker, 2022). A previous study reported that *TaDIR-B1*, a gene associated with resistance to *Fusarium crown rot* in wheat, was first identified using GWAS and QTL mapping (Yang et al., 2021). Similarly, GWAS combined with QTL analysis revealed that *TaMTB*, a gene encoding an m⁶A methyltransferase, is significantly associated with resistance to *wheat yellow mosaic virus* (WYMV) (Zhang et al., 2022c). These approaches provide valuable insights for the mapping and functional characterization of aphid resistance genes in wheat. Nevertheless, most studies on the genetic background and linkage mapping of resistance genes remain at a preliminary stage, and limited progress has been made in fine mapping and functional validation.

6. Non-host resistance: a promising source of durable aphid resistance

Unlike host resistance, which is often governed by specific resistance genes and may be overcome by aphid adaptation, non-host resistance (NHR) refers to the resistance exhibited by all genotypes within a plant species against all isolates of a

given pathogen (Mysore and Ryu, 2004). Because NHR is typically mediated by multiple defense layers and coordinated action of numerous genes, it is widely regarded as one of the most durable and broad-spectrum forms of plant resistance (Mysore and Ryu, 2004; Senthil-Kumar and Mysore, 2013). Similar to the infection phase of pathogens on non-host plants, aphids attempt to penetrate the leaf surface using their stylets regardless of plant species, suggesting that defense responses in non-host plants are induced at the molecular level (Shih et al., 2022; Jaouannet et al., 2014). To date, studies on plant resistance to insects have focused predominantly on the mechanisms underlying compatible plant-insect interactions. Therefore, understanding the mechanisms underlying NHR may provide valuable resources for the development of durable aphid-resistant wheat cultivars.

6.1. The molecular framework associated with non-host resistance against insects

NHR reflects the overall effectiveness of plant defense systems against diverse biotic challenges and should be regarded as a phenomenon arising from the collective action of multiple defense layers, rather than as a distinct branch of plant immune pathways attributable to a single mechanism (Panstruga and Moscou, 2020). Similar to host resistance, NHR involves aphid recognition, activation of defense signaling pathways and defense proteins. However, these responses are often stronger, more rapid, and more effective in non-host interactions, thereby preventing successful aphid colonization.

Aphid non-host resistance begins with aphids being unable to successfully locate, penetrate or utilize host tissues. Aphid stylet penetration of plant tissue is well characterized using the electrical penetration graph (EPG), which can distinguish different types of plant-aphid interactions. For example, in the *Arabidopsis-R. padi* (non-host) interaction, the aphid stylet cannot reach the phloem to feed (Escudero-Martinez et al., 2019). However, in the barley-*M. persicae* (poor-host) interaction, the aphid can successfully penetrate sieve-tube elements to ingest phloem sap, but the level of ingestion is reduction compared with a host interaction, suggesting the plant resistance in different types of interactions likely resided in different plant cell layers (Escudero-Martinez et al., 2019; Tjallingii, 2006). Aphid salivary components are considered signaling molecules that stimulate the activation of defense in non-host plants (Mutti et al., 2008; Hogenhout and Bos, 2011). Silencing the tomato gene *TFT7*, which interacts with the salivary protein Me10 of *M. euphorbiae*, has no effect on host susceptibility to the potato aphid, but enhance the survival and fecundity of the non-host aphid *Aphis gossypii* (Chaudhary et al., 2019). These findings suggest that NHR can operate at multiple stages of aphid feeding and may involve distinct defense barriers in different plant tissues.

Following aphid probing, non-host plants activate defense signaling pathways similar to those involved in host resistance. Among the signaling pathways implicated in NHR, JA is a key regulatory factor of NHR. For example, suppression of JA-dependent defense enabled colonization by previously non-associated herbivore species capable of successful feeding and reproduction, highlighting the role of JA in

shaping herbivore host selection (Bai et al., 2022). When the poor-host plant wheat was fed by *M. persicae*, a strong immune response was activated in the plant, with DEGs significantly enriched in alpha-linolenic acid metabolism and a significant increase in JA content (Liu et al., 2026). Plant proteins are also involved in non-host resistance processes. For instance, the transcriptional response analysis of barley revealed that infestation by *M. persicae* (a non-host interaction) induced a stronger defense response, and several thionin conferred plant resistance to aphids (Escudero-Martinez et al., 2017). Arabidopsis transcriptome analyses identified DEGs during the Arabidopsis-*R. padi* non-host interaction, including toxic protein-related gene vegetative storage protein1 (VSP1), which contributed to non-host resistance against aphids (Jaouannet et al., 2015). A previous study showed that Bowman-Birk trypsin inhibitors (TaBBTI-1), specifically induced by *M. persicae* infestation on wheat (a poor-host interaction), significantly suppressed the fecundity of *S. avenae* and *M. persicae*, as well as the food consumption of *Helicoverpa armigera*, demonstrating broad-spectrum resistance against herbivorous insects (Liu et al., 2026). Collectively, these findings suggest that defense proteins represent important downstream components of aphid NHR.

6.2. Exploiting NHR for durable wheat resistance

Taken together, current evidence suggests that aphid non-host resistance is not mediated by a single defense mechanism but rather by the coordinated action of multiple defense layers. Notably, many of these mechanisms overlap with those involved in host resistance, but they appear to be activated more effectively during non-host interactions. The widespread adoption of genome editing and omics approaches has provided a strong foundation for the precise and efficient utilization of NHR-associated traits in agriculture. Exploiting non-host resistance genes represents a promising strategy for breeding crops with broad-spectrum and durable resistance. Genes involved in NHR have already been successfully applied in plant breeding to confer resistance against pathogens (Zhao et al., 2005; Sendín et al., 2012). Given the mechanistic similarities between aphid-plant and pathogen-plant interactions, NHR is increasingly regarded as a promising strategy for developing broad-spectrum and durable aphid-resistant crops (Enders and Begcy, 2021).

7. Conclusions and perspectives

Current evidence indicates that wheat resistance to aphids is governed by a multilayered defense system involving aphid recognition, immune signaling, physical and chemical defenses, resistance genes, and non-host resistance mechanisms. Aphid salivary proteins play an important role in wheat-aphid interaction, acting either as elicitors that activate plant defenses or as effectors that suppress host immunity. Importantly, resistance is not determined by a single defense component but by the coordinated action of multiple defense layers that collectively influence aphid performance and host suitability. Despite substantial progress, several major bottlenecks continue to hinder the development of durable aphid-resistant wheat. Firstly, only a limited number of aphid salivary effectors have been functionally

characterized, and their molecular targets in wheat remain largely unknown. Secondly, the regulatory networks linking aphid perception to downstream defense responses are still poorly understood. Thirdly, relatively few resistance genes have been effectively utilized in breeding programs, and resistance conferred by individual genes may be overcome by aphid adaptation. Finally, the mechanisms underlying non-host resistance remain insufficiently explored despite their considerable potential for durable resistance breeding.

Future research should move beyond conventional germplasm screening to uncover the core biological mechanisms underlying durable aphid resistance. Priority should be given to the combinatorial action of salivary effectors, rather than single effector proteins. It is likely that aphid salivary proteins function cooperatively to manipulate host plant immunity and hormone signaling. Therefore, future studies should aim to construct “aphid salivary effector networks” through integrated transcriptomics, proteomics, and protein-protein interaction analyses, combined with functional validation in wheat. In parallel, elucidating the molecular basis of NHR may reveal novel defense mechanisms and resistance-associated genes that can be incorporated into wheat breeding programs. The integration of multi-omics approaches, spatial transcriptomics, genome-wide association studies, and functional genomics tools such as RNAi and CRISPR-based genome editing will greatly accelerate the discovery and validation of resistance determinants. Ultimately, combining host resistance genes, endogenous defense pathways, and NHR mechanisms may provide a promising strategy for developing wheat cultivars with durable and broad-spectrum resistance to aphids. Such advances will contribute to environmentally sustainable aphid management and global food security.

Chapter 2

Objectives and thesis outline

1. Objectives

The overall aim of this thesis is to elucidate the molecular mechanisms underlying both host and non-host resistance of wheat to aphids and to provide a theoretical foundation for the development of novel and durable strategies for wheat aphid management. The thesis is organized around the following three objective:

- (1) To explore non-host resistance in wheat to aphids by comparing the adaptability of different aphid species to wheat and the differences in the induced plant defense responses.
- (2) To investigate the molecular mechanism underlying the regulation of rutin biosynthesis in the aphid-resistant wheat cv.HM6172.
- (3) To study the molecular mechanism of the salivary protein Sa6007 of grain aphid *Sitobion avenae* in the induction of wheat defense responses.

2. Thesis outline

Chapter 1 is a review of the research progress on aphid resistance mechanisms (non-host resistance and host resistance), resistance genes and wheat responses mediated by aphid salivary proteins.

In Chapter 3, the adaptability of different aphid species to wheat and the differences in host and poor-host interactions were investigated.

In Chapter 4, the secondary metabolites related to wheat resistance to aphid and the resistance molecular mechanisms were explored on the aphid-resistant wheat variety HM6172.

In Chapter 5, the molecular mechanisms associated to the salivary protein Sa6007 were studied to understand inducing host immune responses.

In Chapter 6, a general discussion and perspectives about wheat resistance to aphids and aphid management were proposed.

Chapter 3

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

Adapted from:

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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 *Acyrtosiphon 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.

Keywords: non-host resistance, wheat, *Myzus persicae*, jasmonic acid, Bowman-Birk trypsin inhibitor

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 (Blackman and Eastop, 2000). 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. 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 (Shih et al., 2022). This induction stimulates the biosynthesis and accumulation of defensive secondary metabolites, such as benzoxazinoids and protease inhibitors (PIs), ultimately impairing insect growth and development (Jia et al., 2024). 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 (Zhou et al., 2009). However, some studies have also 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 (Zhang et al., 2020b; Johnson et al., 2020). Additionally, many resistance loci/genes to aphids have been cloned in host plants, but their durability is limited due to insect adaptation (Radchenko et al., 2022). *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 (Milligan et al., 1998; Nombela et al., 2003; Casteel et al., 2006), but was observed to lose resistance at high soil temperatures (Gabriel et al., 2022). 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 (Radchenko et al., 2022).

Based on the insect's adaptation to the plant, plant-insect interactions can be classified into three types: host, poor-host, and non-host interactions. In a host interaction, the insect can feed and reproduce normally on the plant. In a poor-host interaction, the insect can survive and feed, but its performance is limited. In a non-host interaction, the insect cannot successfully feed or establish on the plant. Non-host resistance represents the most prevalent and durable plant defense strategy against pathogens (Senthil-Kumar et al., 2013; Jones et al., 2024). 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* (Zhao et al., 2005), and transgenic tomato expressing the pepper *Bs2* gene achieved stable resistance against multiple strains of *X. campestris* pv. *vesicatoria* (Sendín et al., 2012). Most aphid species exhibit narrow host specificity confined to a few plant families (Blackman and Eastop, 2000). 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 (Shih et al., 2022; Jaouannet et al., 2014). 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 (Jaouannet et al., 2015). *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 (Jaouannet et al., 2015). 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 (Blackman and Eastop, 2000; Cocu et al., 2005). 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 (Davis and Radcliffe, 2008). Notably, plant thionin genes are strongly upregulated in *M. persicae*-barley poor-host interactions, conferring aphid resistance (Escudero-Martinez et al., 2017).

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 (IWGSC, 2014; Aradottir et al., 2021; Wang et al., 2023). 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 (Shih et al., 2022; Züst and Agrawal, 2016). 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: $60\% \pm 10\%$) on artificial diet (Jah et al., 2012). 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 (Liu et al., 2022). 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 (Hood et al., 1996). 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 (Wang et al., 2007). 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 (cv. Aikang58) 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 hours 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 $65 \pm 5\%$. Raw signals were processed and annotated using Stylet+ software (version 2.3, EPG Systems) according to established waveform classifications as described previously (Prado and Tjallingii, 1994): 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 S. avenae and M. 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 $|\text{Log}_2(\text{fold change})|$ 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. Library construction, RNA sequencing, and bioinformatic analyses were performed by Annoroad Gene Technology Co., Ltd. (Beijing, China). A total of five treatments were established, including *S. avenae* infestation for 6h (Sa6h), *S. avenae* infestation for 24h (Sa24h), *M. persicae* infestation for 6h (Mp6h), *M. persicae* infestation for 24h (Mp24h), and uninfested control groups (0h). Three independent biological replicates were conducted for each treatment.

2.7. Measurement of phytohormone contents in wheat infested with *S. avenae* or *M. persicae*

JA and SA contents in aphid-infested wheat leaves were examined as described previously (Zhang et al., 2023). 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 hours and 24 hours 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 μ L 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 (Zhang et al., 2017a). All primer pairs (Table S3-1) 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 (Livak and Schmittgen, 2002).

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 S3-2. 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. The primers used are listed in Table S3-1. *Agrobacterium* GV3101 carrying pCambia1300: *TaBBTI-1^{sp}-GFP* and

pCAMBIA1300: *GFP* constructs were resuspended in infiltration buffer [100 μ M 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 1mL 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.

2.12. Prokaryotic expression and purification of TaBBTI-1

Recombinant TaBBTI-1 protein was expressed and purified by biotechnology company. Briefly, 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 $\times$ His-tag). The primers used are listed in Table S3-1. 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 (10000 $\times$ 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 $\times$ 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 one hour 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 (Zhang et al., 2020a). Briefly, 6.25, 12.5, 18.25, and 25 μ g of TaBBTI-1 protein (0.5mg/mL) were added to 50 μ L 20 μ g/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 μ L 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) (Zhang et al., 2017a). 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}*) were amplified using FastPfu DNA Polymerase (TransGen Biotech, China) according to the manufacturer's instructions. The primers used are listed in Table S3-1. The pEDV6: *TaBBTI-1^{sp}* recombinant plasmids that carries 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 (Zhang et al., 2022d). 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 MgCl₂ solution (OD₆₀₀ = 1.5). 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 two 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. The primers used are listed in Table S3-1. 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 ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. Host preference and feeding behavior of *S. avenae*, *M. persicae* and *A. 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 (Figure 3-1A). 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 H₂O₂ and callose accumulation at feeding sites (Zhang et al., 2020b; Saheed et al., 2009). Both *S. avenae* and *M. persicae* infestation were found by DAB and aniline blue assays to induce obvious H₂O₂ accumulation and callose deposits in wheat (Figure 3-1B and 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 (Figure 3-1D). 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 ($P \leq 0.01$) (Figure 3-1E). 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.

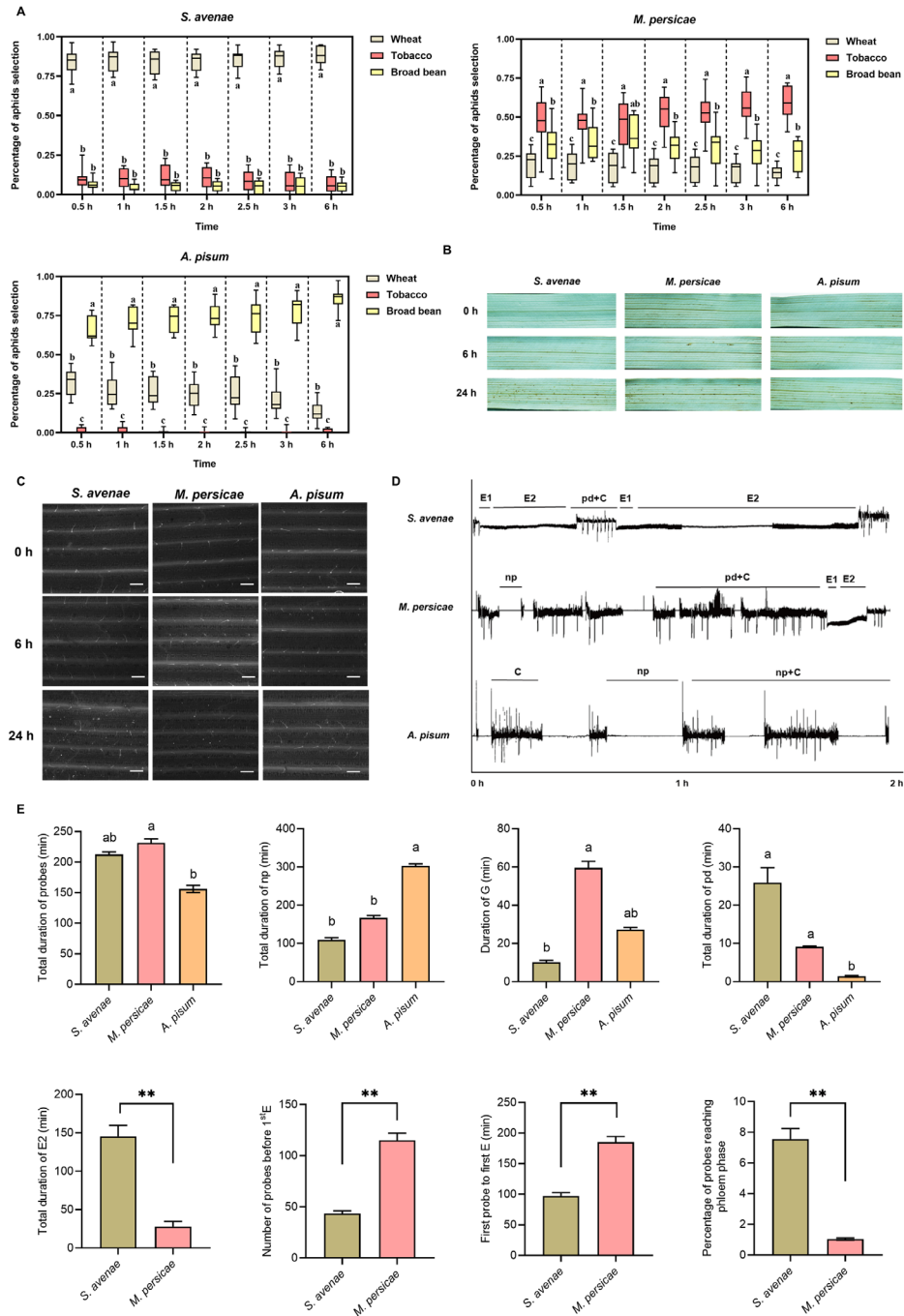


Figure 3-1: Host preference and feeding behaviors of *Sitobion avenae*, *Myzus persicae*, and *Acyrthosiphon pisum* on wheat. (A) Host selection percentages of each aphid species on wheat, tobacco and broad bean plants. Data points and error bars represent means $\pm$ standard error (n

= 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 $\pm$ 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 (** $P \leq 0.01$).

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 (Figure 3-2A). 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 (Figure 3-2B). 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 (Figure 3-2C, 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 *S. avenae*-wheat as a host interaction, *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* (Figure 3-2E). 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 (Figure 3-2F).

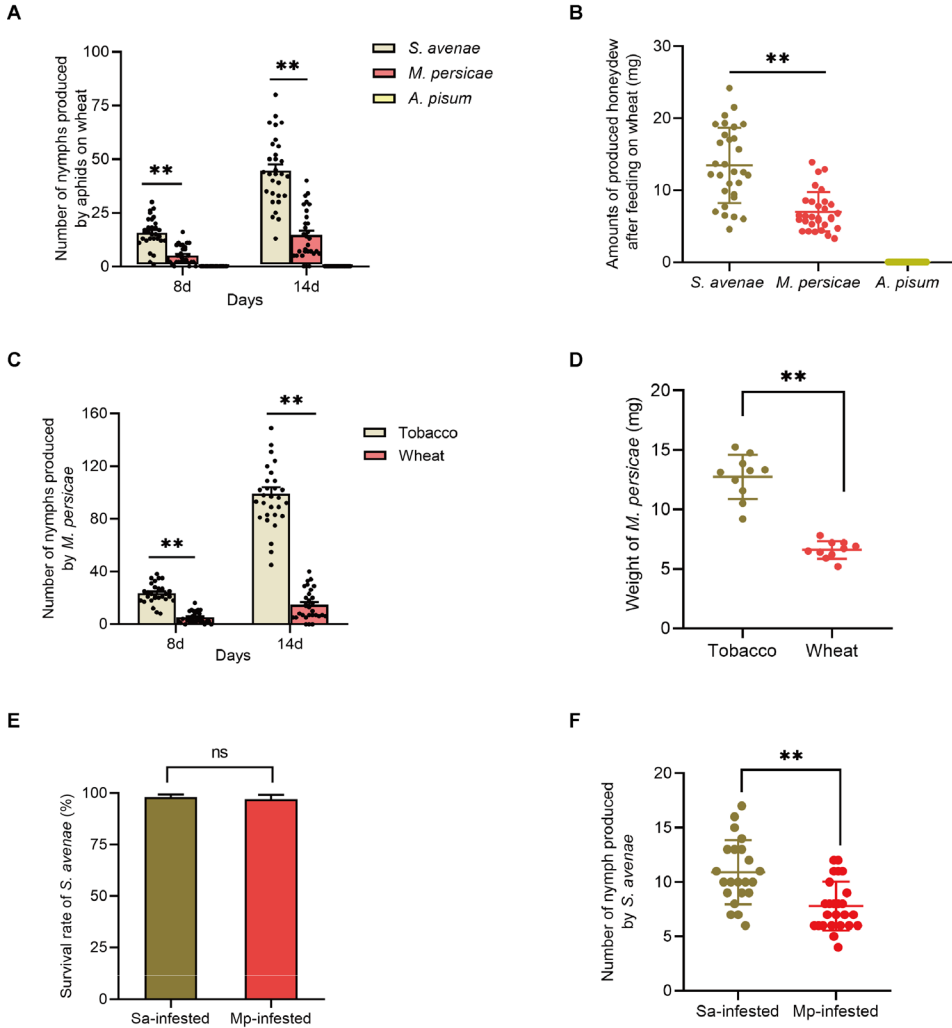


Figure 3-2: Performance of *Sitobion avenae*, *Myzus persicae*, and *Acyrthosiphon pisum* on wheat. (A) Number of nymphs produced by each aphid species after feeding on wheat plants for 8 and 14 days ($n = 30$). (B) Amounts of honeydew excreted by *S. avenae*, *M. persicae* and *A. pisum* after feeding 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* after 14 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. persicae* for 5 days ($n = 20$). (F) Number of nymphs produced by *S. avenae* after feeding on *S. avenae*-preinfested or *M. persicae*-preinfested wheat for 5 days ($n = 20$). Data are presented as mean $\pm$ standard error. Asterisks above the bar indicated significant differences between different groups (** $P \leq 0.01$).

3.3. Comparative transcriptome analysis of wheat host-/ poor-host interactions with *S. avenae* and *M. 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. A total of 102.6 Gb of clean bases were obtained from the 15 leaf samples, and each of these samples contained ≥ 6.3 Gb of data with Q30 quality scores $\geq 95.88\%$ (Table 3-1). 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 (Figure 3-3A). Compared to uninfested wheat plants, 2619 (1598 upregulated, 1021 downregulated) and 13726 (7692 upregulated, 6034 downregulated) DEGs were identified in wheat following 6 h and 24 h of feeding by *S. avenae*, respectively (Figure 3-3B). After *M. persicae* 6h and 24 h post-infestation, 2250 (1842 upregulated, 408 downregulated) and 8179 (5239 upregulated, 2940 downregulated) DEGs were identified when compared to controls (Figure 3-3B).

Table 3-1. Statistical analysis of wheat transcriptomic sequencing data

Sample	Raw reads	Clean reads	Clean bases	Mapping Rate	Q30 (%)	GC-pct(%)
0h-1	47134416	45397466	6.8G	0.8264	96.08	56.89
0h-2	48682430	47142970	7.1G	0.8464	96.24	56.74
0h-3	48807240	47125396	7.1G	0.8444	95.9	56.9
Sa6h-1	47707578	45957252	6.9G	0.8608	95.88	56.83
Sa6h-2	46770806	44950888	6.7G	0.8684	96.21	57.42
Sa6h-3	46350368	44594316	6.7G	0.853	96.08	56.54
Sa24h-1	47831590	45978750	6.9G	0.8215	95.99	56.27
Sa24h-2	46043178	44278558	6.6G	0.8557	96.12	56.32
Sa24h-3	49198374	47431960	7.1G	0.8566	96.34	55.97
Mp6h-1	49329770	46923998	7G	0.8379	95.96	56.82
Mp6h-2	43191810	41769508	6.3G	0.8512	93.25	56.64
Mp6h-3	45673404	44107136	6.6G	0.834	96.13	56.7
Mp24h-1	49028610	47152688	7.1G	0.8558	95.85	56.84
Mp24h-2	48315886	46567836	7G	0.8252	96.22	56.98
Mp24h-3	46244430	44429522	6.7G	0.853	95.94	57.21

KEGG enrichment analysis was further performed to explore the biological functions of DEGs in four comparison groups (Figure 3-3C). The upregulated DEGs in Sa6h vs. 0h and Sa24h vs. 0h 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. 0h and Mp24h vs. 0h 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 (Figure 3-3C).

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. 0h and Mp24h vs. 0h 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 (Figure 3-3D). Among these, a Bowman-Birk trypsin inhibitor (TraesCS3D02G034200), which were designated as TaBBTI-1, showed significant upregulation in both comparison groups (Figure 3-3D). 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 6h and 24h. 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 (Figure 3-3E).

GO enrichment analysis was also conducted to identify the functions of all identified DEGs. In the Sa6h vs. 0h and Sa24h vs. 0h 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 (Figure 3-4). In the Mp6h vs. 0h and Mp24h vs. 0h 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 the molecular function category, the upregulated DEGs were mainly enriched in “DNA-binding transcription factor activity”, “unfolded protein binding” and “terpene synthase activity” (Figure 3-4).

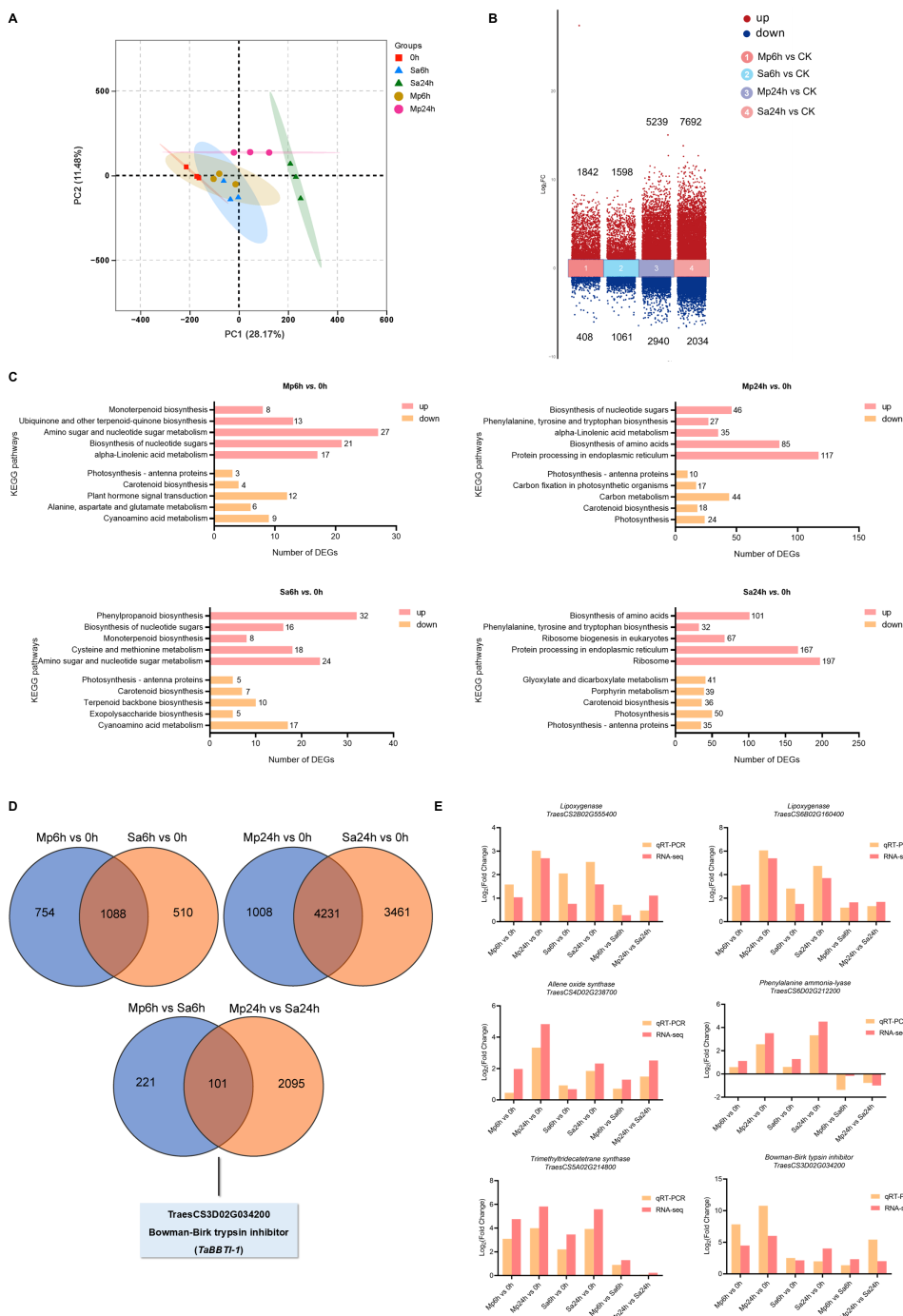


Figure 3-3: Wheat transcriptome profiling in response to interactions with *Sitobion avenae* versus interaction with *Myzus persicae*. (A) Principal component analysis (PCA) of the

expression of unigenes for 0h, 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. 0h, Sa24h vs. 0h, Mp6h vs. 0h, Mp24h vs. 0h comparison groups. (D) Venn diagrams showing the overlap between upregulated DEGs in Sa6h vs. 0h, Sa24h vs. 0h, Mp6h vs. 0h, Mp24h vs. 0h, 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.

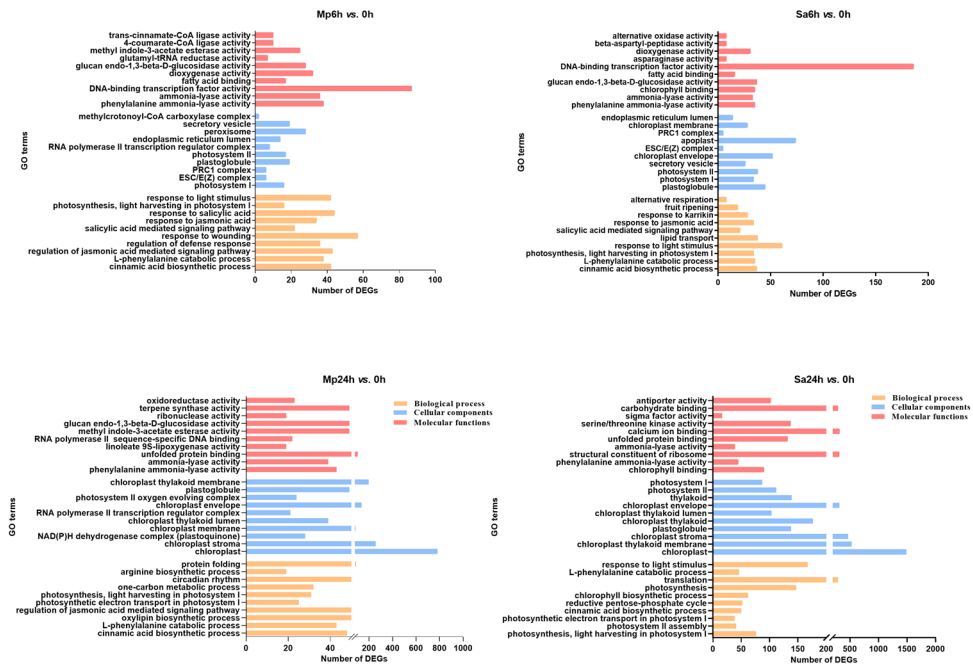


Figure 3-4. GO functional analysis of up-regulated DEGs in Mp6h vs. 0h, Mp24 vs. 0h, Sa6h vs. 0h and Sa24h vs. 0h comparison groups.

3.4. Effect of *M. 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. 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 (OPCLI)*; *acyl coenzyme A oxidase (ACX)*, and *multifunctional protein (MFP)*, were identified in all groups (Figure 3-5A). Of these, 17, 42, 8, and 31 JA-responsive genes were significantly regulated at Mp6h vs. 0h, Mp24h vs. 0h, Sa6h vs. 0h and Sa24h vs. 0h 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. 0h comparison, while the transcript levels of those genes were upregulated 17-, 31-, and 11-fold in the Sa24h vs 0h.

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 ($P \leq 0.05$; Figure 3-5B). 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 ($P \leq 0.05$; Figure 3-5C).

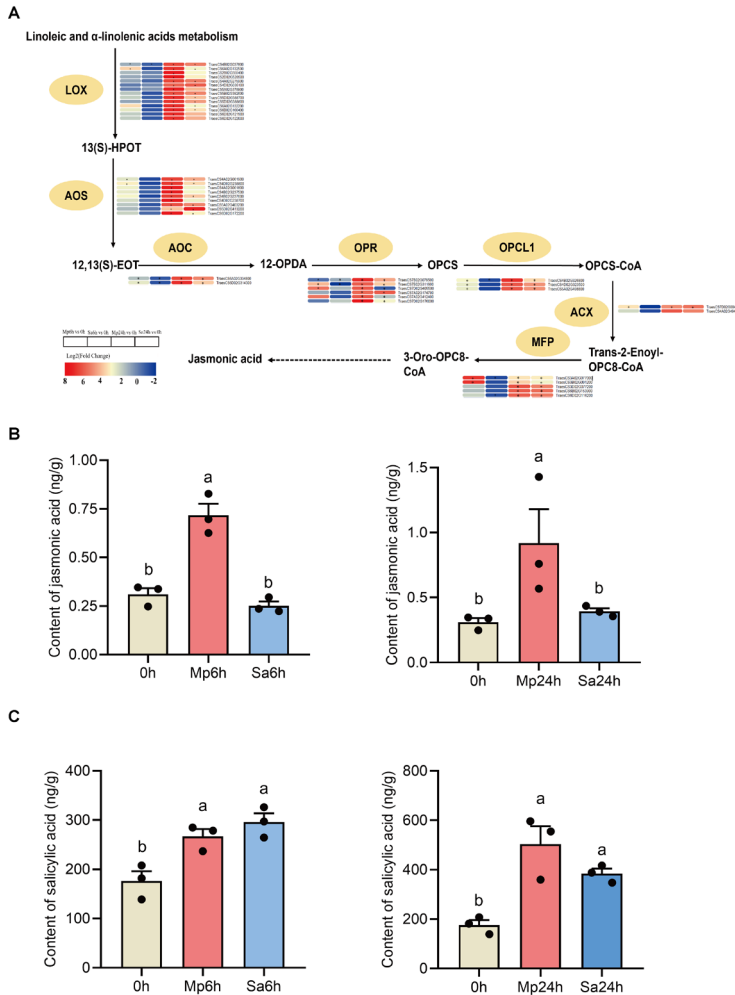


Figure 3-5: Induced jasmonic acid signaling defense pathway in wheat following *Myzus persicae* infestation. (A) Expression profiles of DEGs involved in jasmonic acid biosynthesis. Heatmap shows Log_2 (fold change) values for Mp6h vs 0h, Sa6h vs 0h, Mp24h vs 0h, and Sa24h vs 0h comparisons. Ellipses represent individual genes. Asterisks indicate significant differential expression ($P \leq 0.05$). Color 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 6h and 24h post-infestation. Three replicates were conducted for each treatment. Data are presented as mean $\pm$ standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$).

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 (Figure 3-6), 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 (Figure 3-6), suggesting that JA signaling pathway is associated with the induction of *TaBBTI-1*.

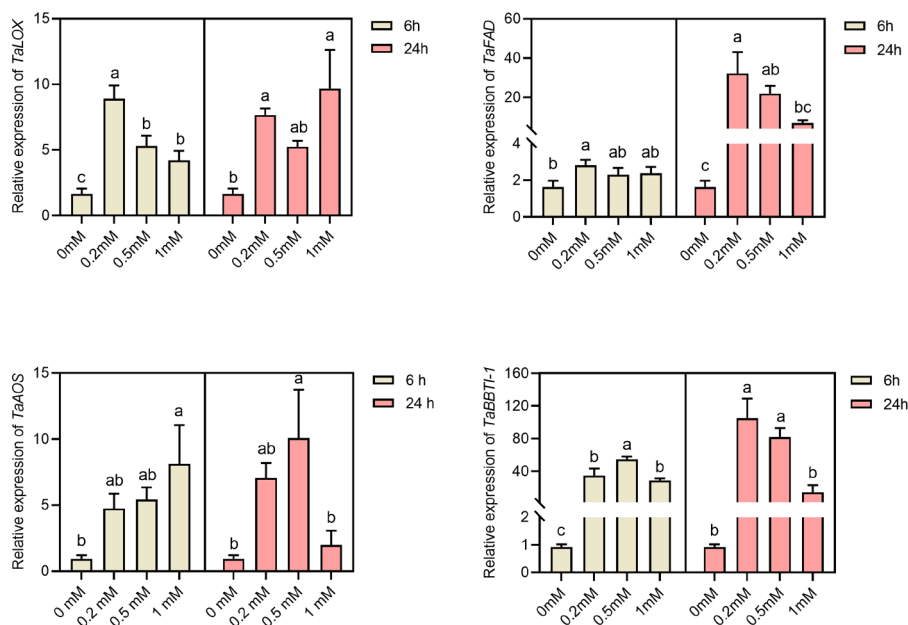


Figure 3-6: Impact of exogenous application of jasmonic acid on gene expression levels. Effects of jasmonic acid treatment on the expression of *TaLOX*, *TaFAD*, *TaAOS* and *TaBBTI-1* in wheat. Data are presented as mean $\pm$ standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$).

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 (Figure 3-7A). The 3D diagram showed that the secondary structure of *TaBBTI-1* consists of one α -helix, eight β -sheets and loops (Figure 3-7B).

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 (Figure 3-7C). 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* (Figure 3-7D).

To verify the trypsin inhibitor activity of TaBBTI-1, the protein was heterologous expressed in *Escherichia coli* using a prokaryotic expression system. SDS-PAGE analysis showed that TaBBTI-1 was successfully purified, exhibiting a molecular weight of approximately 17 kDa (Figure 3-7E). Functional analysis by trypsin inhibition assay demonstrated dose-dependent inhibitory activity that trypsin activity decreased progressively with increasing TaBBTI-1 concentrations (Figure 6F), 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

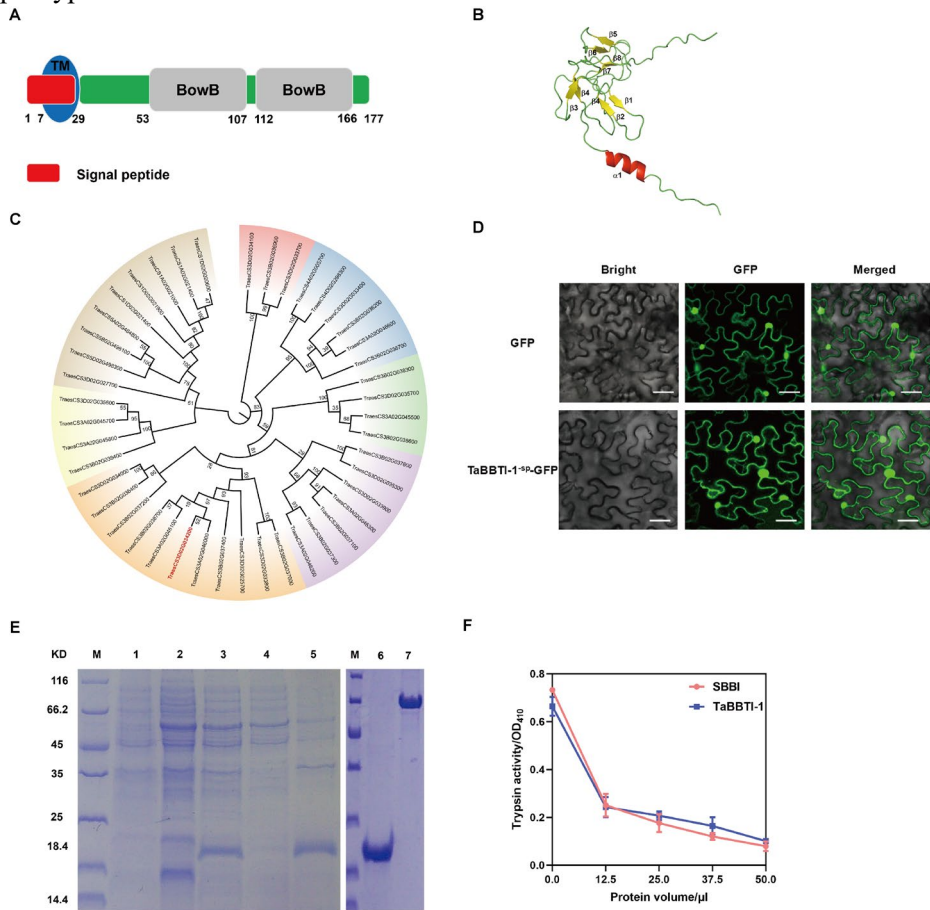


Figure 3-7. Biochemical characterization of TaBBTI-1. (A) Sequence analysis of amino acid sequence of TaBBTI-1. The predicted of the signal peptide, transmembrane domain and two

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 μ m. (E) 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. (F) 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 (SBI) was used as the positive control. Data points and error bars represent means $\pm$ standard error (n = 3)

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 μ g/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 μ g/mL (Figure 3-8A). When feeding on 20 μ g/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 μ g/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 (Figure 3-8B). Similarly, the number of produced nymphs by *M. persicae* 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 (Figure 3-8C). *TaBBTI-1* was then transiently overexpressed in *M. persicae* host plant *N. tabacum* mediated by *A. tumefaciens*. 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 (Figure 3-8D). The results of the leaf area after feeding damages also showed that the *H. armigera* larvae fed on approximately $3.49 \pm 0.73 \text{ cm}^2$ area of the control leaves expressing *GFP*. However, the damaged area of the leaves overexpressed *TaBBTI-1* by *H. armigera* larvae feeding was only $1.32 \pm 0.22 \text{ cm}^2$, which was significantly lower than that of control groups ($P \leq 0.05$) (Figure 3-8E). These results demonstrated that TaBBTI-1 confers broad-spectrum resistance of plants against aphids and lepidopterans.

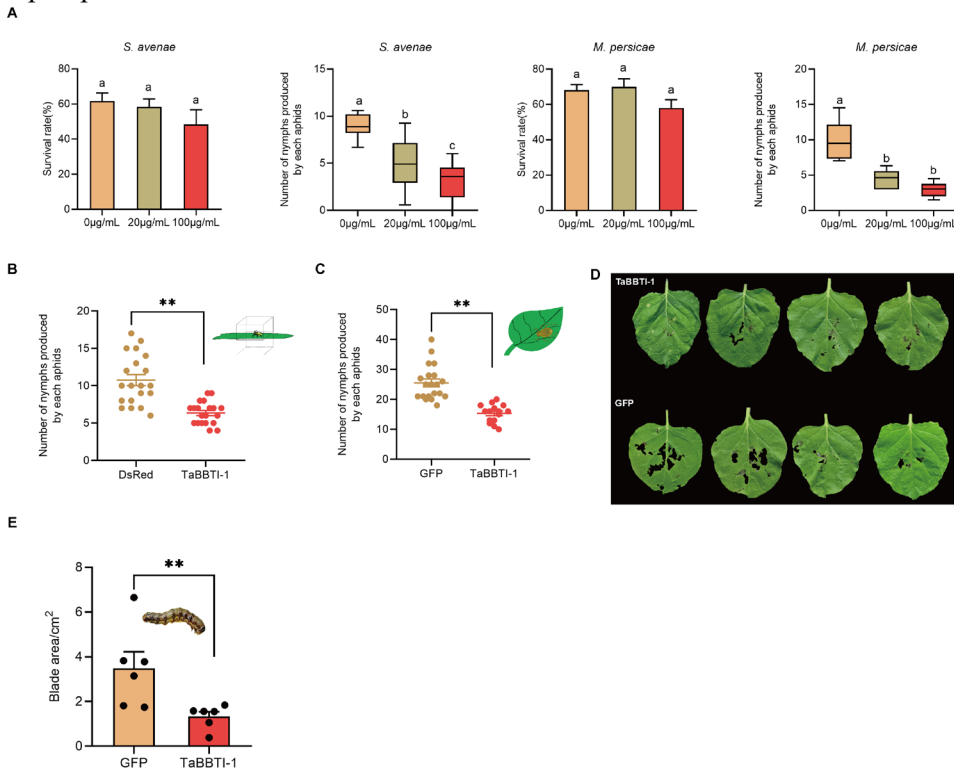


Figure 3-8: Functional characterization of TaBBTI-1 protein in plant resistance against insect herbivores. (A) Survival rate and fecundity of *S. avenae* and *M. persicae* after feeding on artificial diet supplemented with purified TaBBTI-1 protein. Data are presented as mean $\pm$ standard error ($n = 12$). 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-E) 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 $\pm$ standard error ($n = 6$). Asterisks above the bar indicated significant differences between different groups ($**P \leq 0.01$).

4. Discussion

Non-host plant resistance, typically defined as the resistance of plants to incompatible microbial pathogens (Hafez et al., 2020), 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 (Pinheiro et al., 2004). 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 (Davis and Radcliffe, 2008). 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*) (Escudero-Martinez et al., 2017). Aphid probing behavior reflects the adaptability of insect pests to plants during the initial penetration of stylets into plant tissues (Alvarez et al., 2006; Powell et al., 2006). 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 (Huang et al., 2024). 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 (Hu et al., 2008). 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 (Harrington et al., 1986; Schwarzkopf et al., 2013). 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 (Escudero-Martinez et al., 2019). Furthermore, previous studies have reported that continuous aphid feeding induces H₂O₂ and callose accumulation in plants during both compatible and incompatible interactions (Kuśnierczyk et al., 2008; Kachroo et al., 2021). Evident H₂O₂

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 H₂O₂ 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 hours, 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 (Jaouannet et al., 2015). 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 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 (Zhu et al., 2021; Huang et al., 2022), 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 (Yates and Michel, 2018; Züst and Agrawal, 2016). 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 *S. avenae* induces SA pathway genes (e.g., *PAL*, *PR1*) and elevates SA concentrations in wheat (Zhang et al., 2017a; 2020b; Johnson et al., 2020). Furthermore, exogenous SA application enhances resistance in aphid-susceptible wheat against *S. avenae* (Feng et al., 2021). 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* (Huang et al., 2022) and in tea plants fed upon by the citrus black aphid *Toxoptera aurantii* (Luo et al., 2025). Notably, the JA pathway is considered particularly effective against aphids compared with the SA defense pathway (Züst and Agrawal, 2016). Exogenous JA application suppresses aphid populations on tomato and *Brassica* species (Cooper et al., 2004; Ali et al., 2021), whereas inhibiting JA accumulation by silencing *SbLOX3* enhances *M. sacchari*

performance on host plants (Shi et al., 2025). 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) (Zimmerli et al., 2005) and of sweet orange *Citrus sinensis* to citrus canker caused by *Xanthomonas citri* (Rani et al., 2015).

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 (Jaouannet et al., 2015). Additionally, transcriptome analysis of barley infested with *M. persicae* allowed the identification of two *thionin* genes, involved in plant resistance during poor host interactions (Escudero-Martinez et al., 2017). 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 (Birk, 1985). 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 (Deng et al., 2024). Feeding by brown planthopper *Nilaparvata lugens* nymphs induces the upregulation of PI gene expression in resistant rice plants (Weng et al., 2003). Volatiles emitted by rice plants infested with *C. suppressalis* significantly enhance *BBTI* expression and the accumulation of trypsin inhibitors (Yao et al., 2022). Previous studies have also demonstrated that methyl jasmonate application induces proteinase inhibitor synthesis in tomato plants (Farmer and Ryan, 1990). Silencing *OsHI-LOX* significantly reduced both JA and trypsin PI levels in rice, whereas JA treatment increased these inhibitor levels (Zhou et al., 2009). In the present study, exogenous JA treatment significantly upregulated *TaBBTI-1* expression in wheat, indicating that *TaBBTI-1* is responsive to JA and suggesting a potential association between JA signaling and *TaBBTI-1* induction during the poor-host interaction with *M. persicae*. However, the underlying regulatory 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 (Boulter et al., 1989). Addition of SbBBTI, derived from soybean, to an artificial diet significantly reduced population of the potato aphid *M. euphorbiae* (Azzouz et al., 2005). Adding purified BBTI Oh43 to insect diets reduced larval weight and inhibited growth in the fall armyworm *Spodoptera frugiperda* (Johnson et al., 2014). 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 (Zhang et al., 2020a; Liu et al., 2021). 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 (Snoeck et al., 2022; Jones and Dangl, 2006). Several salivary elicitors in aphid saliva such as Mp10, CathB3, and SmCSP4, have been identified as inducers of effective host plant defenses (Bos et al., 2010; Guo et al., 2020; Zhang et al., 2023). 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 (Jones and Dangl, 2006; Mutti et al., 2008; Elzinga and Jander, 2013). The salivary effector Sg2204 of the greenbug *S. graminum* has been shown to suppress both SA- and JA- signaling defense pathways in wheat (Zhang et al., 2022d). 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 (Zhang et al., 2020a). 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* (Harmel et al., 2008; Zhang et al., 2021). 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 significant changes in JA-associated defense responses following *M. persicae* infestation, suggesting the involvement of JA signaling in the wheat response 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*. Together, these findings support the involvement of JA-associated responses and *TaBBTI-1* in poor-host resistance and identify *TaBBTI-1* as a potential candidate for further investigation of broad-spectrum resistance in wheat.

5. Supplementary file

Table S3-1. All primer sequence in this study.

Gene name	Primer sequence (5'-3')	Purpose
qTaPAL	F: GACATCCTCAAGCTCATG R: AGTTCTTCACGGAGGTCT	qPCR
qTaLOX	F: TTCAGCCAGAAGTTGATC R: TCGATGTCAGATGTGTTG	
qTaLOX	F: TGACCAAGACAGCAAGATAA R: CTTCAGGTACGGCATCAC	
qTaAOS	F: ACTTCTACTACTTCCAGGGA R: GACGTTGATGCGGAGGAC	
qTaAOS	F: CACTTCTCCTCGTCTCTC R: AAGTCGTTGAGCATGTTGAA	
qTaTMTT	F: ATGCCATCGTCAAAGAAA R: ATGTCGTAGCCATTGATG	
qTaFAD	F: TCCCATTCCACCTACTGC R: GGACTCACCAATCCGAGA	
qTaBBTI	F: TACCATCCTGCTGATGCTTG R: CACTCGAAAACCTGGTCCAT	
β -Actin	F: GGAAAATCAGTCTCGGTTTCTAG R: TCATACAGCAGGCAAGCAC	Gene clone
TaBBTI	F: ATGGGGATGAAGCGATGC R: CTAGTTACCGCCGGCGGCGACAGCC	
TaBBI- KpnI- XbaI	F: cggggtaccATGGCCATTCTCCTGCC R: tgctctagaGTTACCGCCGGCGGCGACAGCC	Transient overexpression
attB-TaBBI	ggggacaagttgtacaaaaagcaggttcGCCATTCTCCTGCCGAG ggggaccactttgtacaagaaagctgggtcGTTACCGCCGGCGGCGAC AGCC	Delivering by T3SS

Table S3-2 All TaBBTI amino acid sequences used for phylogenetic tree construction:

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DSPTCTKSNPPTCRCGDEVDKCAPTCKTCLPSRPRPSRRVCLDSYFGPFPPACTPKAV
AAGGN

>TraesCS3B02G037300

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GDHNEALAKAGDHQDSVAAAAEEEEKPWECCDQVIPADVPLRGPRAVRYNMQE
LRGVHLEVPVPRQLQRLVQRLPRSQVHRGRRQRR

>TraesCS3D02G027700

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RVCNDWYHGFPGAKCTAGGN

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>TraesCS3D02G033400

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EPSGFNPAFHVCNDQYHGDGPPTCAKEERPWTCCDRTVCTKSFPPTCRCLDEVEEC
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THQVDVAGAGN

>TraesCS4A02G005700

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GGPPRRRRRRRRRAYVVG
SGLATNEGGEETPPDKVMEAEK
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GQGLGADATALGAPMK
KVLEEDERPWACCDDTHCLR
VLPRACLCRDTVAQCASASQHC
DEVSPGRYVCLDIH
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AAAVNRGRQPWKCCDQ
PICSGWKYPVCECADEVDE
CAPTCKSCVPSKANATRKVC
EDTFIGKAGPGCTEKPWK
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DEVQCAPTCKTCLPALLHP

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SGYMCRDAYRGYPGPKRM
L

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Chapter 4

The TaAHL19-TaF3GT module regulates wheat resistance to aphids through activating rutin biosynthesis

Adapted from:

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Liu, X.B., Wang, Q., Zhou, J.Y., Wu, P.P., Liu, H., Zhang, Y.M., Tan, X.L., Fan, J., Francis, F., Liu, Y., Zhan, Y.D., Liu, Y.X., Fu, Y., Chen, J.L., Zhang, Y. (2026) The TaAHL19-TaF3GT module regulate wheat resistance to aphids through activating flavonoid rutin biosynthesis. Submitted to *Advanced Science* (Under review)

Abstract

Aphids are devastating pest that seriously affect the quality and yield of wheat worldwide. However, the mechanisms underlying wheat resistance to aphids remain poorly understood. Through subsequent evaluations of aphid performance and feeding behavior, this study identified *Triticum aestivum* Hanmai 6172 (cv. HM6172) and *T. aestivum* Nongda 399 (cv. ND399) as aphid-resistant and aphid-susceptible wheat varieties, respectively. cv. HM6172 was found to exhibit antibiotic resistance to aphids. Comparative transcriptomic analysis of resistant and susceptible wheat varieties following aphid infestation revealed that feeding on cv. HM6172 specifically upregulated genes associated with the phenylpropanoid and flavonoid biosynthesis pathways. Targeted metabolomics further revealed that aphid feeding significantly induced the accumulation of flavonoids including rutin and luteolin in resistant wheat cv. HM6172, whereas no significant changes were detected in the susceptible variety ND399. Additionally, dietary supplementation with rutin significantly reduced the fecundity of *Sitobion avenae*, *Schizaphis graminum*, and *Myzus persicae*, and exerted lethal effects on leaf-chewing insects *Spodoptera frugiperda* larvae. A rutin biosynthesis-related gene, UDP-glucose: flavonol 3-O-glucosyltransferase (*TaF3GT*), was specifically induced by aphid infestation in cv. HM6172. Silencing *TaF3GT* via virus-induced gene silencing (VIGS) significantly decreased rutin content and consequently enhanced plant susceptibility to aphids. Furthermore, an AT-hook motif nuclear-localized protein 19 (*TaAHL19*) of wheat was found to directly bind to the promoter of the *TaF3GT* and promote its expression. Overexpression of *TaAHL19* significantly increased both aphid resistance and rutin accumulation in wheat. These results provided novel insights into the molecular basis of wheat resistance to aphids and thereby offered promising genetic resources for breeding improvement.

Keywords: wheat, aphid resistance, rutin biosynthesis, transcriptional factor *TaAHL19*

1. Introduction

The previous chapter demonstrated that poor-host interactions constitute a valuable source of broad-spectrum defence mechanisms and identified TaBBTI-1 as an important defence component associated with non-host resistance. However, because non-host resistance originates from incompatible plant–insect interactions, its direct application in wheat breeding remains challenging. Therefore, exploring naturally occurring resistance within wheat germplasm represents a more practical approach for identifying genetic resources and molecular regulators that can be readily utilized in aphid-resistant breeding.

The utilization of host-plant resistance is recognized as a cost-effective and environmentally sustainable strategy for pest management. To cope with biotic stresses like insect herbivory, plants have evolved sophisticated resistance mechanisms, which can be categorized into constitutive and inducible types (War et al., 2012). Inducible plant resistance, activated upon herbivore attack, are primarily mediated by phytohormonal signaling pathways involving salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Züst et al., 2016). The activation of these pathways leads to the accumulation of various plant secondary metabolites, which function as crucial defense compounds by reducing plant digestibility and directly deterring pests including flavonoids, cardenolides, glucosinolates, alkaloids, and benzoxazinoids (Erb and Reymond, 2019; Ullah et al., 2019, 2022).

Flavonoids, a major class of polyphenolic compounds, play a critical role in enhancing plant resistance against herbivores and pathogens (Nan et al., 2016; Shen et al., 2022). For instance, resistant rice varieties were found to accumulate higher levels of flavonoids such as schaftoside, isoschaftoside, and neoschaftoside compared to susceptible varieties, contributing to resistance against the brown planthopper *Nilaparvata lugens* (Grayer et al., 1994). Similarly, exogenous application of flavonoid extracts increased larval mortality of the fall armyworm *Spodoptera frugiperda* in maize (Lima et al., 2021), while spraying kaempferide and apigenin on wheat spikes enhanced resistance to Fusarium head blight caused by *Fusarium graminearum* (Su et al., 2020). Artificial applications of the extract of total flavonoids to rice have also increased resistance to *N. lugens* (Dai et al., 2019). The role of flavonoids in plant defense is closely linked to the flavonoid biosynthesis pathway, which is regulated by key enzymes including phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavonol 3-hydroxylase (F3H), and flavonol 3'-hydroxylase (F3'H). Genes associated with flavonoid biosynthesis including *CHS*, *CHI*, *F3H* and *F3'H* were significantly induced in plants infested by stem canker *Fusarium zanthoxyli* (Wang et al., 2020; Li et al., 2021). Infection of *Phytophthora capsici* specifically upregulated multiple flavonoid-related genes of cucumber in resistant varieties, but not in susceptible ones (Mansfeld et al., 2017). The flavanone 3-hydroxylase (*OsF3H*) gene, directly regulated by growth regulating factor 8 (*OsGRF8*), mediated resistance to the brown planthopper (*Nilaparvata lugens*) by influencing total flavonoid contents (Dai et al., 2019). Silencing *CsUGT89A2* in

tea plants enhanced *Toxoptera aurantia* reproductive rates by reducing flavonoid glycoside levels, indicating that *CsUGT89A2* enhances aphid resistance in tea plants through the regulation of flavonoid glycoside (Zhou et al., 2025b).

Breeding and utilizing aphid-resistant cultivars are crucial strategy for aphid management (Zhang et al., 2022b). Wheat resistance to aphids can be classified into physical and chemical resistance. Trichomes can serve as a physical barrier to enhance wheat resistance to *R. padi* (Correa et al., 2020), and the wild plants exhibits stronger physical defense (Batyreshina et al., 2020). Previous research revealed secondary metabolites play an important role in plant resistance, such as volatile organic compounds (VOCs), benzoxazinoids, phenolic and flavonoid (Borg et al., 2025a; Batyrshina et al., 2020). Saponin, a diglycosyl flavone, was a key factor in reducing the survival rate of *S. avenae* on *Triticum monococcum* (Borg et al., 2025b). The content of flavonoids was positively correlated with the developmental duration of *S. avenae* on different resistant wheat varieties (Zhang et al., 2022a). Similarly, the wheat resistance was correlated with *S. avenae*-induced accumulation of specific flavonoids, namely apigenin, luteolin, (+)-catechin, and (–)-epicatechin in the *Triticale* (Czerniewic et al., 2017). Additionally, resistance genes against aphid species such as *S. graminum* (*Gb1*, *Gby*) and Russia wheat aphid *Diuraphis noxia* (*Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn6* and *Dnx*) have been identified in wheat genetic resources and deployed in breeding programs (Radchenko et al., 2022). One resistance gene against *S. avenae*, designated RA-1 was characterized from *Triticum durum* C273 (Liu et al., 2012). Although some aphid-resistant wheat varieties have been identified and several resistance genes have been mapped in several wheat varieties, the mechanisms underlying resistance remain unclear.

To identify additional aphid-resistant wheat varieties and clarify their resistance mechanisms, the commercial cultivar Hanmai 6172 (cv. HM6172), widely grown in China, was characterized in this study as an aphid-resistant variety exhibiting antibiosis. Combining transcriptomic and metabolomic profiling revealed that the flavonoid rutin is specifically induced in the antibiosis-resistant wheat variety HM6172 following aphid infestation. Functional analysis demonstrated that rutin confers broad-spectrum resistance in wheat against both phloem-feeding and leaf-chewing herbivores. The AT-hook transcription factor *TaAHL19* was demonstrated to bind the promoter of the UDP: flavonol 3-O-glucosyltransferase gene (*TaF3GT*) and activate its expression, resulting in enhanced rutin biosynthesis and wheat resistance to cereal aphids.

2. Materials and methods

2.1. Insects and plants

Clones of *S. avenae* and *S. graminum* were originally collected from a wheat field in Langfang City, Hebei Province, China (39°31'N, 116°42'E), and clone of green peach aphid *M. persicae* was obtained from a tobacco field in Kunming City, Yunnan Province, China (24°23'N, 102°30'E). All aphid clones were reared for over 3 years on their corresponding host plants, including wheat (cv. Zhongmai175) and tobacco,

under laboratory conditions (16 h light/8 h dark cycle, $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$). Fall armyworm *Spodoptera frugiperda* larvae were collected from a maize field in Dehong Prefecture, Yunnan Province, China ($24^{\circ}43'\text{N}$, $98^{\circ}43'\text{E}$) and reared for more than ten generations on a bean-based artificial diet, as described previously (José et al., 2019) in an incubator at $26 \pm 2^{\circ}\text{C}$, $70 \pm 5\%$ RH, with a 16L: 8D photoperiod.

2.2. Age-stage life table of *S. avenae* on aphid-resistant and -susceptible wheat varieties

Wheat plants were transferred to a 2 mL centrifuge tube filled with water and placed in a 9 cm diameter Petri dish. A wingless adult aphid was placed on each plant to produce nymphs for 24 h. After 24 h, all aphids were removed, except for one nymph on each plant. Nymph development was observed daily until adulthood, with daily counting and removal of produced nymphs until all adult aphids died. Seedlings were replaced every 5 days during the experiment. Sixty biological replicates were performed for each variety, and the experiments were conducted in an incubator (16 h light/8 h dark cycle, $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$). The age-stage life table theory was employed to analyze raw life table data of aphids feeding on the resistant variety HM6172 and the susceptible variety ND399 using the TWOSEX-MS Chart program. Population parameters, including intrinsic rate of increase (r_m), net reproductive rate (R_0), finite rate of increase (λ), mean generation time (T), age-specific survival rate (l_x), age-specific fecundity (m_x), age-stage-specific survival rate (s_{xj}), and age-specific female fecundity (f_{xj}) were estimated using the bootstrap method with 100,000 replicates.

2.3. Monitoring of feeding behavior of *S. avenae* on aphid-resistant and -susceptible wheat varieties

The feeding behavior of *S. avenae* on the aphid-resistant variety HM6172 and aphid-susceptible variety ND399 was monitored using an electrical penetration graph (EPG) device (Giga-8 DC, Wageningen, The Netherlands). The EPG assay was performed as described in Section 2.5 of Chapter 3. Each aphid and plant constituted one biological replicate and were used only once. Fifteen biological replicates were performed for each treatment. The resulting waveforms, including np, C, pd, E1, E2, and G were analyzed using Stylet+ software as described previously (Prado and Tjallingii, 1994).

2.4. Wheat plants infested with aphids in the field

The aphid-resistant wheat variety HM6172 and aphid-susceptible wheat variety ND399 were naturally infested with aphids in the field conditions (Baoding City, Hebei Province, China ($38^{\circ}48'\text{N}$, $115^{\circ}24'\text{E}$)). At the filling stage, flag leaves were collected from wheat plants infested with approximately 100 aphids. After removing all aphids gently with a soft brush, the leaves were cut using sterilized scissors and immediately frozen in liquid nitrogen after washed twice with distilled water. All samples were stored at -80°C until further analysis. Wheat plants without aphid infestation enclosed in a nylon net bags were used as controls.

2.5. Whole-transcriptome sequencing of wheat in response to aphid infestation

Total RNA was extracted from aphid-infested wheat leaves using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocols. The quantity and quality of RNA were evaluated using a DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA), and RNA integrity was verified through RNA Nano 6000 assay kit and the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Subsequently, cDNA libraries were constructed according to the Illumina NEBNext Ultra RNA Library Prep Kit protocol and sequenced on an Illumina NovaSeq 6000 platform (pair-end mode). Clean reads were mapped to the wheat reference genome (version 1.0; ftp.ensemblgenomes.org/pub/plants/current/fasta/triticum_aestivum/) using HISAT2 (v2.0.5). Differential expression analysis was conducted with DESeq2 (v1.20.0) across four comparison groups: THM6172 vs CHM6172, TND399 vs CND399, CHM6172 vs CND399, and THM6172 vs TND399. Genes with an adjusted P -value ≤ 0.05 and $|\text{Log}_2(\text{Fold Change})| \geq 1$ were considered significantly differentially expressed (DEGs). Functional enrichment analysis of DEGs for Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using the ClusterProfiler package (R v3.8.1). Library construction, RNA sequencing, and subsequent bioinformatic analyses were performed by Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China). Non-aphid-infested wheat plants and wild-type plants were used as the respective control groups. All RNA-seq experiments included three biological replicates.

2.6. Quantification of flavonoid metabolites in aphid-infested wheat plants using ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

Flavonoid metabolite profiling and quantification were performed by Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China). Briefly, 100 mg fresh wheat flag leaves infested with aphids were collected in the field as described above. After being washed three times with distilled water, the leaf tissues were immediately ground into a fine powder in liquid nitrogen and then extracted with a methanol-acetonitrile-water mixture (2:2:1, v/v). The supernatant was collected after centrifugation at $13000 \times g$ for 20 min at 4°C and stored at -20°C until further analysis.

Flavonoid metabolites were analyzed using an ACQUITY UPLC I-Class system coupled with a 5500 QTRAP mass spectrometer (AB Sciex, Framingham, MA, USA). All flavonoid compounds (>98% purity) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Separation was carried out on a BEH C18 column ($1.7\ \mu\text{m}$, $2.1 \times 150\ \text{mm}$) with a 15 min linear gradient at a flow rate of $400\ \mu\text{L}/\text{min}$. The mobile phase consisted of eluent A (0.1% formic acid) and eluent B (0.1% acetic acid). The gradient program was set as follows: 95% A / 5% B at 0 min; 80% A / 20% B at 3 min; 55% A / 45% B at 9 min; 2% A / 98% B at 13 min; and 95% A / 5% B at 15 min. MS detection was performed in both positive and negative ionization modes with spray voltages of 5500 V and 4500 V, respectively. Data acquisition was conducted

in multiple reaction monitoring (MRM) mode. Raw data were processed using MultiQuant software (version 3.0.3). Significantly altered flavonoids in infested leaves were identified using Student's *t*-test compared with non-infested controls, with a *p*-value < 0.05 considered statistically significant. Each treatment included six biological replicates, with non-infested leaves serving as controls.

2.7. Correlation analysis of transcriptomic and metabolomic data

Correlation analysis was performed to examine the correlation between DEGs and differential flavonoid metabolites. The correlation coefficient ($|r|$) was calculated using the *cor* function in the R package. Correlation network diagrams were subsequently constructed based on DEGs involved in flavonoid biosynthesis and flavonoid metabolites with $|r| > 0.6$.

2.8. Effects of feeding on artificial diet containing rutin on herbivore performance

To evaluate the effects of rutin on insect performance, rutin (Sigma-Aldrich Chemical) was added into artificial diets (20% sucrose solution) with final concentrations of 0.2, 0.4, and 0.8 mg/L. After 1 h of starvation, five fourth-instar aphids of three aphid species *S. avenae*, *S. graminum*, and *M. persicae* were respectively transferred to individual feeding device (Zhang et al., 2021) and reared under controlled environment conditions (16L: 8D photoperiod; 20 ± 1 °C; relative humidity: $60\% \pm 10\%$). The number of surviving aphids and the number of nymphs produced by aphids was recorded daily for 7 days. A total of twelve replicates were performed for each treatment. The effects of dietary rutin on the performance of leaf-chewing herbivores *S. frugiperda* were also investigated. Eight first-instar larvae were reared on an artificial diet (José et al., 2019) supplemented with rutin at 0.2, 2, and 4 mg/L under controlled environment conditions (L: D = 16 h: 8 h; 20 ± 1 °C; relative humidity: $60\% \pm 5\%$). Larval body weight and survival rate were measured after 10 days. Ten biological replicates were conducted for each treatment. Artificial diets without rutin were set as controls.

2.9. Sequence analysis of TaF3GT and TaAHL19

Phylogenetic analysis of TaAHL19 amino acid sequences was constructed using the neighbor-joining method in MEGA7.0 software. The bootstrap values were calculated as a percentage from over 1000 replications. All amino acid sequences used in this analysis were listed in Table S4-1. Potential binding sites for *AHL* transcriptional factors on the promoter sequence of *TaF3GT* were predicted using the JASPAR database (<https://jaspar.genereg.net/analysis>).

2.10. Barley stripe mosaic virus (BSMV)-mediated gene silencing

BSMV-mediated gene silencing in aphid-resistance wheat HM6172 was performed as previously described (Yuan et al., 2011). A specific fragment of *TaF3GT* was

cloned into the pCa- γ bLIC vector using ligation-independent cloning (LIC) to generate the pCa- γ bTaF3GT construct. The primers used are listed in Table S4-2. The pCa- γ bTaF3GT construct and BSMV components pCaBS- α and pCaBS- β , was independently transformed into *A. tumefaciens* strain GV3101. The bacterial cultures were resuspended in an infiltration buffer (100 μ M acetosyringone, 10 mM MES, and 10 mM MgCl₂) to an OD₆₀₀ of 0.6. For agroinfiltration, equal volumes of the three bacterial suspensions (carrying pCaBS- α , pCaBS- β , and pCa- γ bTaF3GT) were mixed and inoculated into the leaves of 4-week-old *N. benthamiana* plants using a 1 mL syringe. At 7 days post-infiltration, the infiltrated leaves were harvested and ground in a 20 mM sodium-phosphate buffer (pH 7.2) containing 1% (w/v) celite. The resulting solution was mechanically inoculated onto leaves of two-leaf-stage wheat seedlings. Systemic leaves were collected at 10 dpi for RNA extraction, and the silencing efficiency of TaF3GT was examined using RT-qPCR. The BSMV:00 (empty vector) and BSMV: TaPDS constructs served as the negative and positive controls, respectively. Three biological replicates were performed for each treatment.

2.11. Yeast One-Hybrid (Y1H) assay

Y1H assay was carried out using the MATCHMAKER One-Hybrid System (Clontech, CA, USA) following the manufacturer's instructions. The coding sequence of TaAHL19 was cloned into pGADT7-Rec2 to generate the prey construct, and the regulatory region of the TaF3GT promoter (proTaF3GT) was inserted into pHIS2 construct the reporter (bait) vector. The primers used are listed in Table S4-2. Subsequently, the Y187 yeast strain was co-transformed with pGADT7-AHL19 and pHIS2-proTaF3GT and cultured on SD/-Trp/-Leu/-His medium supplemented with 50- mM 3-amino-1,2,4-triazole (3-AT) to assess the protein-DNA interaction. Yeast cells co-transformed with pHIS2-p53 and pGAD53m served as a positive control.

2.12. Dual- Luciferase (LUC) reporter assay

The promoter sequence of TaF3GT and the coding sequence of TaAHL19 were respectively cloned into pGreenII0800-LUC and pGreenII 62-SK to construct the reporter and effector vectors. The primers used are listed in Table S4-2. The 35S: TaAHL19 effector plasmid and the proTaF3GT reporter plasmid were co-transformed into *A. tumefaciens* strain GV3101 and subsequently infiltrated into leaves of *N. benthamiana* as described previously. Luciferase (LUC) and Renilla luciferase (REN) activities were measured at 72 h post-infiltration using the Dual-Luciferase Assay System (Promega, USA). Luminescence signals were captured and quantified with a Promega GloMax® 2020 imaging system. Each treatment included six biological replicates.

2.13. Construction of TaAHL19-overexpressing wheat lines

The full open reading frame (ORF) of TaAHL19 was cloned into the pWMB111 expression vector. The primers used are listed in Table S4-2. This vector contains a selectable marker cassette with the *Bar* gene driven by the *Zea mays* ubiquitin (*ZmUbi*) promoter. Wheat transformation was performed by Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. The pWMB111-TaAHL19 construct was

then introduced into *A. tumefaciens* strain EHA105 for wheat transformation according to a previously study (Wang et al., 2022a). Transgenic Plantlets with well-developed root systems were transplanted into pots and cultivated in a controlled environment chamber under a 16-h light/8-h dark photoperiod at 24 ± 1 °C. Total genomic DNA was extracted following a Plant Genomic DNA Kit (TransGen Biotech, China) according to the manufacturer's protocols to detect the transgenic plants. PCR amplification was performed in 20 μ L reaction containing about 200 ng genomic DNA, 10 μ L Taq MasterMix (TransGen Biotech, China), 1 μ L of each primer (10 mL) and distilled water. The primers used are listed in Table S1. Wild-type (Fielder) and two transgenic wheat lines (OE2 and OE6) from the T3 generation were cultivated in an environmental chamber under controlled conditions (16-h light/8-h dark cycle; 24 ± 1 °C). After plant maturity, agronomic traits were evaluated by measuring grain length, width, and thousand-grain weight using randomly sampled grains from each line. Ten biological replicates were performed for each treatment.

2.14. Aphid bioassays on *TaAHL19*-overexpressing wheat lines

To evaluate the effects of *TaAHL19* overexpression on aphid performance, five newly emerged apterous adults of *S. avenae* were introduced onto wheat leaves of individual WT or OE plants at the two-leaf stage and confined using clip cages as described above. The number of nymphs produced by the aphids on each plant was recorded daily for 5 days. This experiment included eighteen biological replicates per wheat line. All assays were conducted under controlled environmental conditions (16-h light/8-h dark cycle; 20 ± 1 °C).

2.15. Quantification of rutin content in wheat plants

The content of rutin was measured in dry samples of *TaF3GT*-silencing wheat leaves and transgenic wheat leaves by high-performance liquid chromatography (HPLC) as described previously (Yao et al., 2020), with minor modifications. Briefly, 100 mg of ground tissue was extracted with 1 mL of methanol. The extract was concentrated by rotary evaporation to 0.2 mL and the supernatant was filtered through a 0.45 μ m organic phase filter and analyzed using a WD-C18 column. The mobile phase consisted of methanol and water/acetic acid (45: 55, v/v). A 10 μ L injection volume was used, and a sample was eluted at a flow rate of 0.6 mL/min and detected at a wavelength of 360 nm. The rutin concentration in each sample was determined using a standard curve.

2.16. RT-qPCR analysis

RT-qPCR was performed to validate the RNA-seq results by profiling expression levels of nine genes involved in phenylpropanoid biosynthesis and flavonoid biosynthetic pathways in *S. avenae*-infested cv. HM6172 and cv. ND399 wheat leaves. The expression dynamics of *TaAHL19* and *TaF3GT* were detected in wheat leaves post 6 h, 12h, 24h and 48 h aphid infestation. The expression profiles of JA-associated defense genes lipoxygenase (*TaLOX*), allene oxide synthase (*TaAOS*), and *TaAHL19* and *TaF3GT* were examined following exogenous JA treatment. Additionally, the expression level of *TaAHL19* and *TaF3GT* were detected in *TaAHL19*-overexpressing

wheat lines and WT wheat line. Total RNA extraction and qRT-PCR were performed as described in Section 2.9 of Chapter 3. Three biological replicates were performed for each treatment. Each biological replicate consisted of three technical replicates.

2.17. Statistical analysis

All statistical analyses were performed using IBM-SPSS Statistics 26.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were examined using Student's *t*-test (for comparisons between two groups) or one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (for multiple group comparisons). The Mann-Whitney U-test was used for analyzing data that did not follow a normal distribution. Differences were considered statistically significant at $P < 0.05$, with $P < 0.01$ indicating highly significance.

3. Results

3.1. Effect of cv. HM6172 and cv. ND399 on the growth of *S. avenae*

As shown in Table 4-1, the intrinsic rate of increase (r_m), finite rate of increase (λ), net reproductive rate (R_0), and mean generation time (T) of *S. avenae* on cv. HM6172 were significantly lower than those on cv. ND399 ($P < 0.001$). As shown in Figure 4-1A, the variable s_{xj} of *S. avenae* was observed in the two wheat varieties. The death of adult aphids occurred on day 40 in cv. ND399 and day 32 in cv. HM6172, indicating a lower survival rate of aphids on HM6172 than on cv. ND399. The l_x , f_{xj} , m_x , and age-specific maternity ($l_x m_x$) were further analyzed to compare the survival rate and fecundity of *S. avenae* on these two wheat varieties (Figure 4-1B). In particular, l_x curves showed a rapid decline between days 12 and 32 on cv. HM6172, whereas a steady decline was observed on cv. ND399. The m_x and $l_x m_x$ values demonstrated that cv. HM6172 had lower fecundity than cv. ND399. The pattern of age-stage-specific life expectancy (e_{xj}) revealed a shorter life expectancy for cv. HM6172 than for cv. ND399 (Figure 4-1C). In addition, the age-stage-specific reproductivity (v_{xj}) demonstrated that aphids produced fewer nymphs for a shorter duration on cv. HM6172 than on cv. ND399 (Figure 4-1D).

Table 4-1. Population parameters of *S. avenae* on cv. HM6172 and cv. ND399

Population parameter	cv. HM6172	cv. ND399	<i>P</i> -value
Intrinsic rate of increase (r_m)	0.2536±0.0073	0.2796 ± 0.0047	<0.01**
Finite rate of increase (λ)	1.2885±0.0094	1.3226±0.0062	<0.01**
Net reproductive rate (R_0)	20.1585±1.2161	40.3857±2.1139	<0.01**
Mean generation time (T)	11.8501±0.2508	13.2237±0.1484	<0.01**

The data in the table are mean ± standard error, ** indicates significant difference between different treatments (paired Bootstrap test, B=100,000, $P < 0.01$).

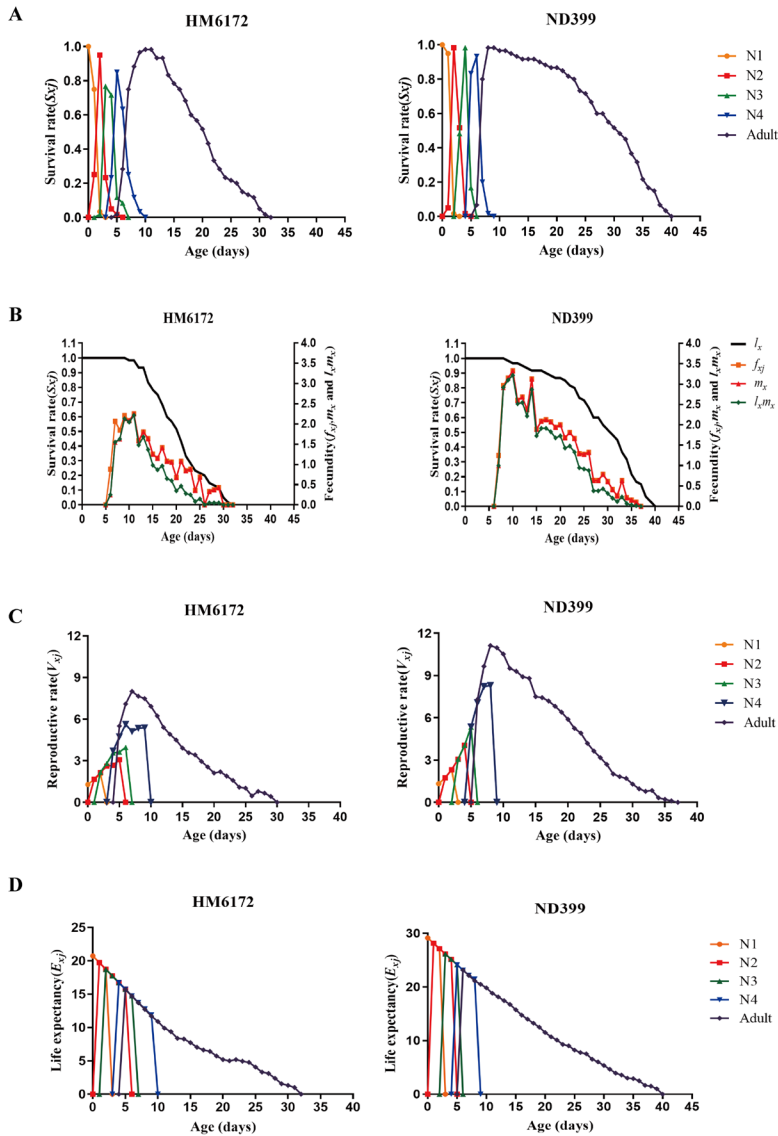


Figure 4-1: Effect of cv. HM6172 and cv. ND399 on the growth of *S. avenae*. (A) Age-stage survival rate (s_{xj}) of *S. avenae* on the aphid-resistant wheat variety HM6172 and the aphid-susceptible wheat variety ND399. (B) Age-specific survival rate (l_x), female age-specific fecundity (f_{xj}), age-specific fecundity (m_x), age-specific maternity ($l_x m_x$) of *S. avenae* on the aphid-resistant wheat variety HM6172 and the aphid-susceptible wheat variety ND399. (C) Age-stage specific reproductive (v_{xj}) of *S. avenae* on the aphid-resistant wheat variety HM6172 and the aphid-susceptible wheat variety ND399. (D) Age-stage specific life expectancy (e_{xj}) of *S. avenae* on the aphid-resistant wheat variety HM6172 and the aphid-susceptible wheat variety ND399.

3.2. Feeding behavior of *S. avenae* on cv. HM6172 and cv. ND399

As representative EPG waveforms in Figure 4-2A, the feeding behavior of *S. avenae* varied greatly when feeding on cv. HM6172 and cv. ND399. The total duration of probes was significantly higher in cv. HM6172 than in cv. ND399 ($P = 0.003$), but the total duration of pd was significantly lower in cv. HM6172 than in cv. ND399 ($P = 0.007$; Figure 4-2B). The time from the first probe to the first ingestion and the probe duration before the first ingestion showed that aphids had a longer latency to reach phloem in cv. HM6172 ($P = 0.026$ and 0.039 , respectively). Furthermore, there was a significant reduction in the duration of phloem ingestion (E2) in cv. HM6172 compared to cv. ND399 ($P = 0.001$), but an opposite trend was observed for the duration of xylem ingestion ($P = 0.026$). These findings indicated that antibiosis resistance of cv. HM6172 leads to deterrent effects on the feeding behavior of *S. avenae*.

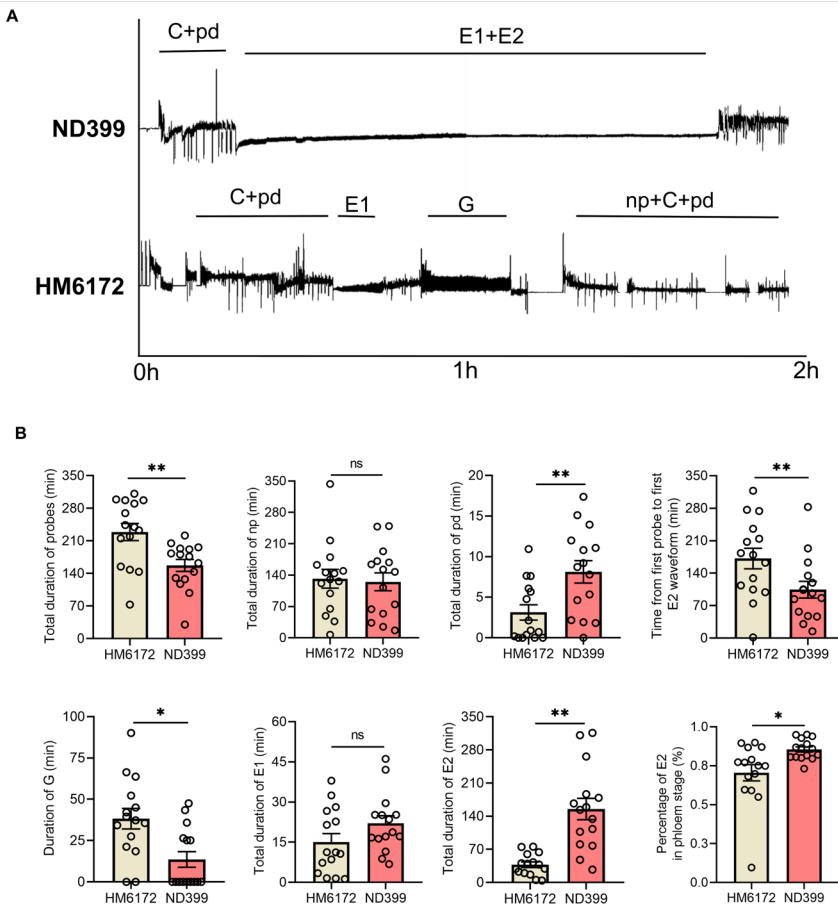


Figure 4-2: cv. HM6172 suppressed the feeding behavior of *S. avenae*. (A) Representative EPG waveforms of aphids on aphid-resistant wheat cv. HM6172 and aphid-susceptible wheat cv. ND399. np: not probing; C: stylet probing; E1: salivation; E2: phloem ingestion; G: xylem ingestion. (B) Effects of different resistance level of wheat on the feeding behaviour of *S. avenae* ($n = 15$, Student's *t* test). Data are from mean $\pm$ standard error. Asterisks above the bars indicate significant differences between different groups (ns: not significant; * $P < 0.05$; ** $P < 0.01$).

3.3. Comparative transcriptome profiling of cv. HM6172 and cv. ND399 in response to aphid infestation

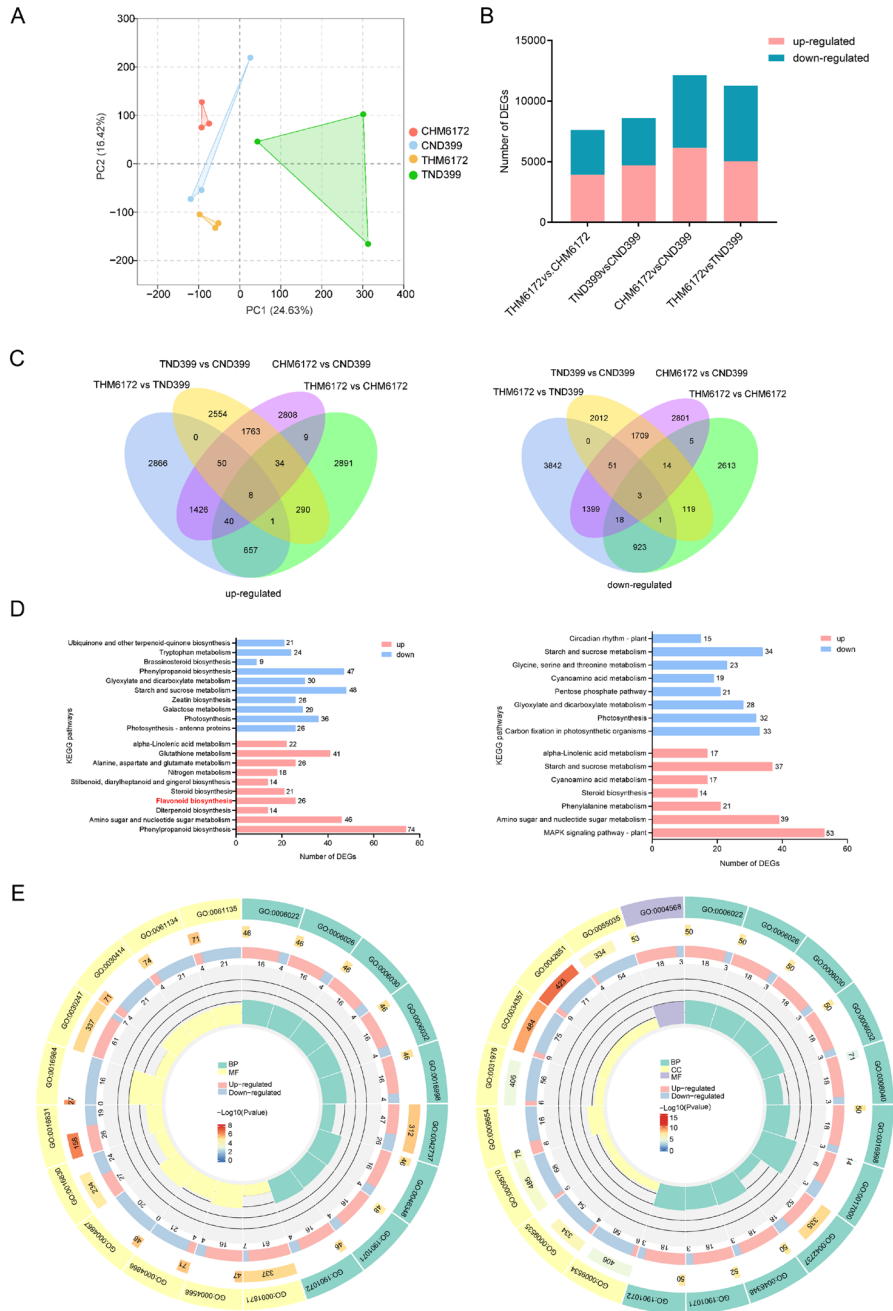
To elucidate the molecular mechanisms of wheat resistance to aphids, we performed transcriptome profiling of the resistant cv. HM6172 and susceptible cv. ND399 lines under aphid infestation. High-quality sequencing data were obtained (Table 4-2), with principal component analysis (PCA) confirming strong reproducibility among biological replicates (Figure 4-3A). After aphid infestation, 7626 DEGs (3930 upregulated and 3696 downregulated) were identified by comparing infested and non-infested cv. HM6172 (THM6172 vs. CHM6172), whereas 8609 DEGs (4700 upregulated and 3909 downregulated) were identified by comparing infested and non-infested cv. ND399 (TND399 vs. CND399) (Figure 4-3B). In cross-comparisons, 12238 DEGs (6238 upregulated and 6000 downregulated) were identified by comparing non-infested cv. HM6172 and non-infested cv. ND399, whereas 11285 DEGs (5048 upregulated and 6237 downregulated) were identified by comparing infested cv. HM6172 and infested cv. ND399 (Figure 4-3B). A core set of 11 DEGs was consistently differentially regulated across all four comparisons (Figure 4-3C).

Table 4-2. Statistical analysis of wheat transcriptomic sequencing data

Sample	Raw reads	Clean reads	Clean bases	Error rate	Q20	Q30	GC-pct
CHM6172-1	45781218	44478836	6.67G	0.03	97.43	93.12	56.72
CHM6172-2	47898792	46612718	6.99G	0.03	97.37	93	56.83
CHM6172-3	44359590	43052082	6.46G	0.03	97.52	93.32	56.89
CND399-1	44341260	42852236	6.43G	0.02	97.91	94.34	56.83
CND399-2	40846726	39521930	5.93G	0.03	97.54	93.38	57.32
CND399-3	41180678	39727872	5.96G	0.03	97.82	94.17	56.74
THM6172-1	42422264	40930792	6.14G	0.03	97.5	93.16	55.9
THM6172-2	46217276	44613644	6.69G	0.02	97.94	94.38	55.56
THM6172-3	43006178	41946706	6.29G	0.03	97.77	94	55.88
TND399-1	46740020	45338612	6.8G	0.03	97.48	93.21	56.35
TND399-2	43368388	40365830	6.05G	0.03	97.76	93.5	49.4
TND399-3	57143096	55008936	8.25G	0.02	98.23	94.74	51.5

GO and KEGG enrichment analysis highlighted distinct functional categories for the DEGs in each comparison. KEGG enrichment analysis revealed that upregulated DEGs in THM6172 vs. CHM6172 comparison were mainly enriched in phenylpropanoid biosynthesis, flavonoid biosynthesis, diterpenoid biosynthesis, steroid biosynthesis, and amino sugar and nucleotide sugar metabolism (Figure 4-3D). In TND399 vs. CND399 comparison, upregulated DEGs were related to mitogen-activated protein kinase (MAPK) signaling pathway, steroid biosynthesis, amino

sugar and nucleotide sugar metabolism, phenylalanine metabolism, and cyanoamino acid metabolism (Figure 4-3D). Although seven induced pathways were shared between THM6172 vs. CHM6172 and TND399 vs. CND399 comparisons (e.g., plant MAPK signaling pathway, amino sugar and nucleotide sugar metabolism, phenylalanine metabolism, α -linolenic acid metabolism), upregulated DEGs in the resistant cv. HM6172 infested with aphids were notably enriched in phenylpropanoid and flavonoid biosynthesis, pathways that were absent in the aphid-infested susceptible variety ND399. These results suggested that phenylpropanoid and flavonoid biosynthesis are key pathways associated with aphid resistance in wheat. The most significantly enriched GO terms in THM6172 vs. CHM6172 comparison were “drug catabolic process” and “aminoglycan metabolic process” in BP, “photosystem II” in CC, and “carboxy-lyase activity” in MF (Figure 4-3E). In the TND399 vs. CND399 comparison, the top enriched terms were “drug catabolic process” (BP), “thylakoid membrane” (CC), and “chitinase activity” (MF) (Figure 4-3E).



comparison and TND399 vs. CND399 comparison. (E) The top 20 enriched GO terms for each of the three classes in the THM6172 vs. CHM6172 comparison and TND399 vs. CND399 comparison.

3.4. Validation of RNA-seq data

To validate the RNA-seq results, we selected nine differentially expressed genes (DEGs) for RT-qPCR analysis to examine their expression in cv. HM6172 and cv. ND399 following aphid infestation. The selected genes comprised key enzymes from the phenylpropanoid pathway including *TaPAL* (TraesCS2D02G377200), *TaBGLU* (TraesCS5B02G294600), *TaF6'H* (TraesCS6D02G270700), *TaF5H* (TraesCS2A02G490100), *CYP98A3* (TraesCS3A02G331800), *TaHCT* (TraesCS6A02G022100), and *TaCAD4* (TraesCS6B02G201600), as well as *TaCHS* (TraesCS2A02G527700) and *TaF3'H* (TraesCS6B02G354500) from the flavonoid biosynthesis pathway. The results showed that the expression patterns of selected genes were highly consistent with the transcriptome data (Figure 4-4). This strong correlation confirmed the reliability of our RNA-seq analysis.

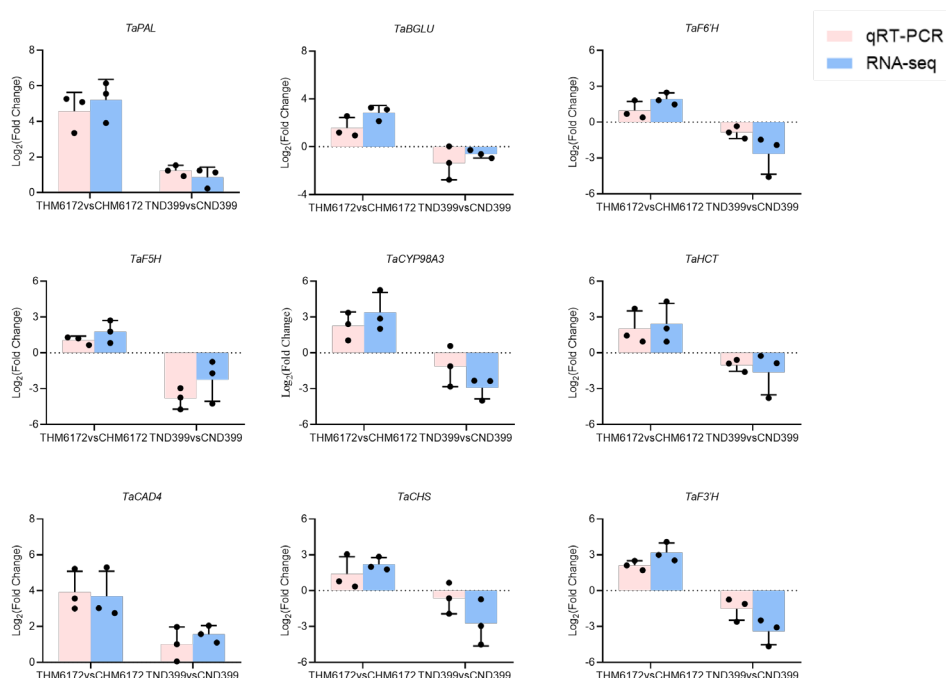


Figure 4-4: Comparison of expression levels of key genes in RNA-seq and qRT-PCR analysis. Pink bar represents the fold change in relative gene expression determined by quantitative real-time PCR, and blue bar represents the fold change in relative gene expression determined by transcriptome data. Data are from mean $\pm$ standard error.

3.5. Wheat defense response dependent on jasmonic acid

As JA is important phytohormone in transducing the activation of plant defense systems, DEGs involved in JA signaling pathways were analyzed. In THM6172 vs. CHM6172 comparison, 18 significantly upregulated genes associated with JA biosynthesis were identified, including lipoxygenase (*TaLOX*), allene oxide synthase (*TaAOS*), 12-oxophytodienoate reductase (*TaOPR*), peroxisomal 3-ketoacyl-CoA thiolase 3 (*TaPKT3*), and jasmonic acid carboxyl methyltransferase (*TaJMT*) (Figure 4-5A). However, only three significantly downregulated genes, mainly encoding *TaOPR*, acyl-CoA oxidase 1 (*TaACX1*) and *TaJMT* were identified in TND399 vs. CND399 comparison. The transcript levels of *TaLOX* and *TaJMT* were > 6- and 4-fold higher, respectively, in infested cv. HM6172 than those in non-infested cv. HM6172, whereas it was ~2- and 10-fold lower in infested cv. ND399 than those in non-infested cv. ND399. The contents of JA in cv. HM6172 and cv. ND399 wheat leaves following infestation by *S. avenae* were quantified using LC-MS/MS. We found that JA content in the resistant wheat variety HM6172 was 32-fold greater than that of aphid-susceptible variety ND399 (Figure 4-5B). Additionally, after aphid feeding, the JA content in cv. HM6172 wheat plants was significantly higher than that in cv. ND399.

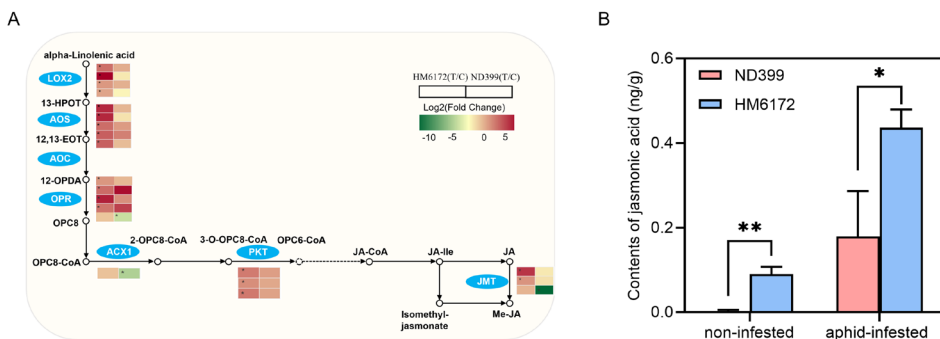


Figure 4-5: Induced jasmonic acid signaling defense pathway in wheat following aphid infestation. (A) Expression profiles of DEGs involved in jasmonic acid biosynthesis. Heatmap shows Log₂ (fold change) values for THM6172 vs CHM6172 and TND399 vs CND399 comparisons. (B) Endogenous jasmonic acid levels in wheat leaves infested with *S. avenae*. Three replicates were conducted for each treatment. Data are presented as mean $\pm$ standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$).

3.6. Flavonoid biosynthesis pathways in wheat plants induced by aphid infestation

Transcriptome analysis revealed that aphid infestation specifically activated the phenylpropanoid and flavonoid biosynthetic pathways in the resistant cv. HM6172, but not in the susceptible cv. ND399. In cv. HM6172, the expression of key phenylpropanoid pathway genes, including *TaPAL*, 4-coumarate-CoA ligase (*Ta4CL*), cinnamoyl-CoA reductase (*TaCCR*), cinnamic acid 4-hydroxylase (*C4H*), cinnamyl-

alcohol dehydrogenase (*TaCAD*), O-methyltransferase (*TaOMT*), ferulate 5-hydroxylase (*FAH*), and peroxidases were significantly upregulated upon infestation (Figure 4-6). In contrast, no significant enrichment of DEGs in this pathway was observed in cv. ND399. Similarly, the downstream flavonoid pathway was also specifically induced in cv. HM6172. The expression of two key genes, *TaCHS*, chalcone isomerase (*TaCHI*) were markedly upregulated in infested cv. HM6172 but downregulated in infested cv. ND399. For instance, *TaCHS* expression increased approximately 6-fold in cv. HM6172 but decreased about 8-fold in cv. ND399 after infestation. Additionally, flavonol 3'-hydroxylase (*TaF3'H*) was activated only in cv. HM6172. These results demonstrated that the coordinated induction of phenylpropanoid and flavonoid biosynthesis is a key molecular response associated with aphid resistance in wheat.

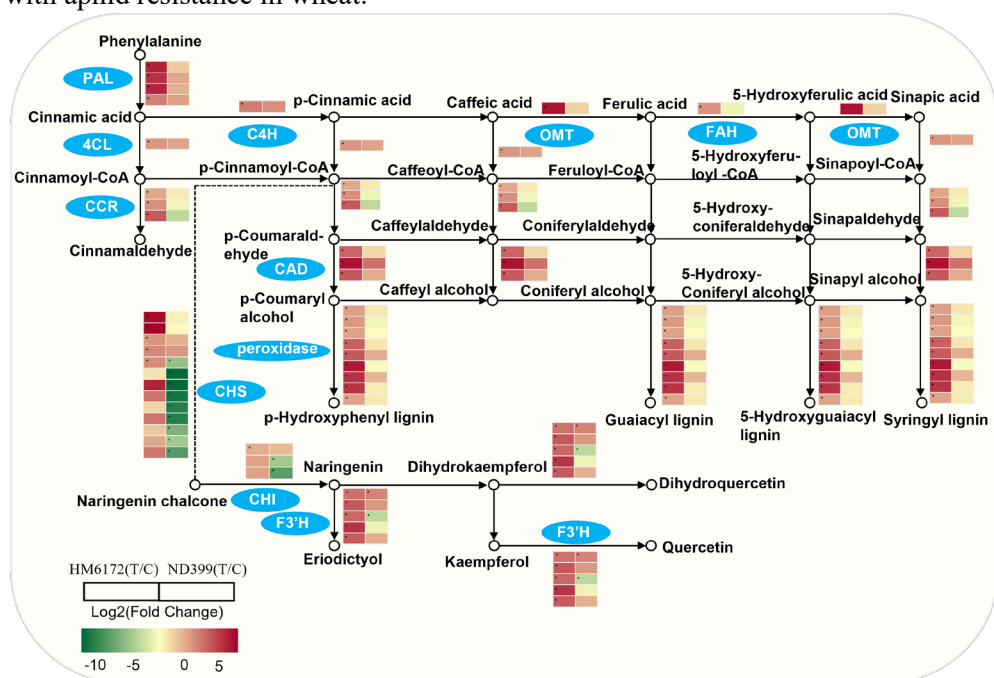


Figure 4-6: Expression profiles of genes involved in the phenylpropane and flavonoid biosynthetic pathways in the resistant wheat cv. HM6172 and susceptible wheat cv. ND399 in response to aphid infestation. Circles represent metabolites, ellipse represent genes (* $P < 0.05$). The heatmap scale ranges from -10 to 5 on a log2 fold change.

3.7. Metabolic profiles of flavonoids in cv. HM6172 and cv. ND399 in response to aphid infestation

Target metabolomic profiling revealed distinct flavonoid accumulation patterns in the resistant (cv. HM6172) and susceptible (cv. ND399) wheat following aphid infestation. A total of 23 and 21 flavonoids were detected in cv. HM6172 and cv. ND399, respectively. Principal component analysis showed clear separation between

infested and control samples of each variety, indicating that aphid feeding significantly altered the flavonoid profiles (Figure 4-7A).

In the resistant cv. HM6172, infestation of *S. avenae* significantly upregulated seven flavonoids, including ferulic acid, phenylalanine, rutin, luteolin, chrysin, and luteolin-7-O-glucoside, while glycitin was downregulated (Table 4-3; Figure 4-7B). Notably, rutin exhibited the most pronounced increase in infested cv. HM6172 plants, with a 17.11-fold upregulation compared to controls, yet it was undetectable in both non-infested (CND399) and infested plants (TND399) of the susceptible cv. ND399. Aphid infestation on susceptible cv. ND399 led to a distinct metabolic profile that the contents of glycitin and *p*-coumaric acid were significantly elevated, whereas chrysin, luteolin-7-O-glucoside, apigenin, genistein, biochanin A, puerarin, butin, and catechin were significantly reduced (Table 4-3). Intriguingly, glycitin, chrysin, and luteolin-7-O-glucoside displayed opposite accumulation trends in cv. HM6172 and cv. ND399 in response to aphid infestation.

Table 4-3. Differential metabolites in cv. HM6172 and cv. ND399 in response to aphid infestation

Metabolite	cv. HM6172		cv. ND399	
	Fold change	<i>P</i> -value	Fold change	<i>P</i> -value
Ferulic acid	8.469307717	2.96364E-06		
phenylalanine	2.339836767	5.42359E-06		
Glycitin	0.043002958	6.4166E-06	2.706687	0.00473
Rutin	17.11538611	1.99628E-05	-	-
Luteolin	5.221437954	0.000270255	-	-
Chrysin	1.574681996	0.009972722	0.276251	0.004816
Luteolin-7-O glucoside	1.969153696	0.019354255	0.418227263	0.009740252
Apigenin	-	-	0.334877488	0.000146991
Genistein	-	-	0.457098965	0.000328847
Biochanin A	-	-	0.162956014	0.0021645
Puerarin	-	-	0.343934305	0.004197231
Butin	-	-	0.880770107	0.007183085
Catechin	-	-	0.786922784	0.007515098
<i>p</i> -Coumaric acid	-	-	1.711121638	0.014124035

–: Indicates that in this treatment group, the metabolome was not detected or the fold of difference was not significant

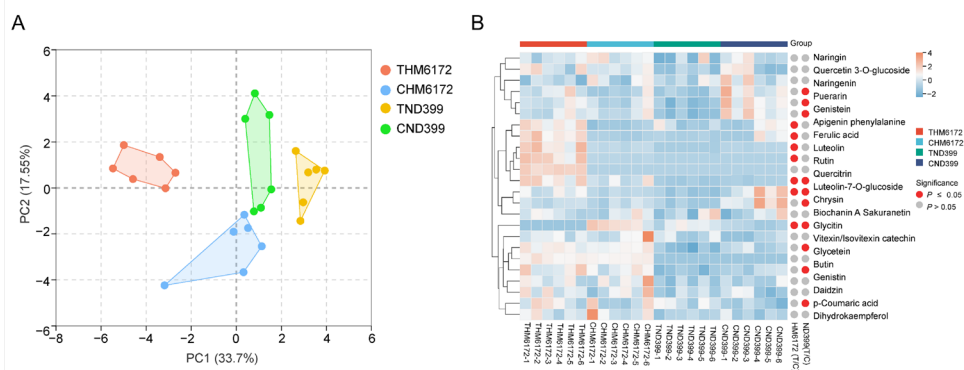


Figure 4-7: Overview of flavonoids profiles of cv. HM6172 and cv. ND399 in response to aphid. (A) PCA of the flavonoids in different comparisons, CHM6172, THM6172, CND399, and TND399. (B) Heatmap comparing the flavonoids in different comparisons. Yellow indicates highly expressed flavonoids and blue indicated less expressed flavonoids.

3.8. Effects of rutin on the performance of three aphid species and *Spodoptera frugiperda*

To assess the potential role of rutin in conferring wheat resistance, we examined its direct effects on the performance of three aphid species (*S. avenae*, *S. graminum*, and *M. persicae*) and the leaf-chewing herbivore *S. frugiperda* when incorporated into artificial diets. The results showed that feeding on artificial diet with rutin at concentrations of 0.2, 0.4 and 0.8 mg/L had no significant effects on the survival of any aphid species, but significantly inhibited the fecundity of all three aphid species in a dose-dependent manner (Figure 4-8). The number of nymphs produced by *S. avenae*, *S. graminum*, and *M. persicae* was significantly reduced when treated with 0.2 mg/L rutin, and the inhibitory effect enhanced with increasing concentration of rutin. For example, the mean number of nymphs produced by each *S. avenae* adult over 7 days decreased from 5.53 at 0.2 mg/L to 2.78 at 0.8 mg/L.

In addition, rutin adversely affected the performance of polyphagous pest *S. frugiperda*. The larval survival rate decreased to $67.19\% \pm 7.81\%$ after feeding on an artificial diet with 0.2 mg/L rutin and was further reduced to only $45.31\% \pm 7.44\%$ at 4 mg/L. The body weight of *S. frugiperda* was also significantly reduced when feeding on artificial diet supplemented with rutin (Figure 4-8). These results demonstrated that rutin, which is highly induced in cv. HM6172 upon aphid infestation, confers broad-spectrum resistance against both phloem-feeding and leaf-chewing insect pests.

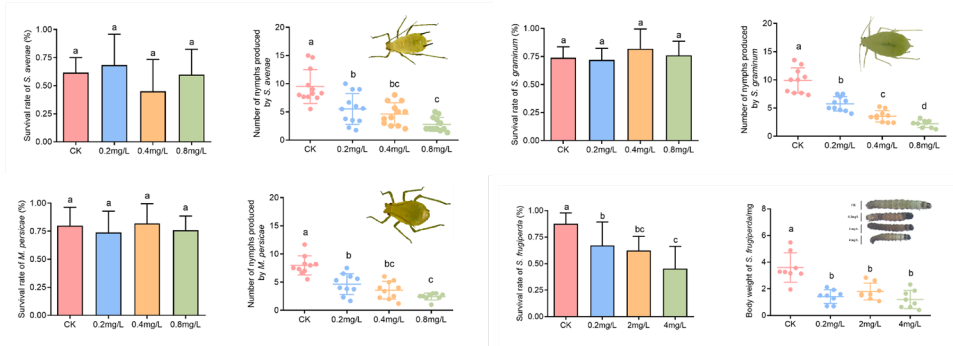


Figure 4-8: Effect of artificial diet supplemented with rutin on the survival rate and fecundity of *S. avenae*, *S. graminum*, *M. persicae* ($n = 12$), and the survival rate and body weight of larvae of *S. frugiperda* ($n = 10$). Data are from mean $\pm$ standard error and a, b, c indicate significant differences between different treatment (One-way ANOVA followed by Duncan's multiple comparisons at $P < 0.05$).

3.9. Correlation analysis of dynamics transcriptomes and flavonoid metabolites in wheat leaves infested with aphids

A total of 29 DEGs associated with rutin synthesis were identified across all groups. These included *TaPAL*, *Ta4CL*, *TaC4H*, *TaCHS*, *TaCHI*, *flavonol 3'-TaF3'H* and *UDP-glucose: flavonol 3-O-glucosyltransferase (TaF3GT)* were identified in all groups (Figure 4-9A). Among them, 18 genes were significantly upregulated in the THM6172 vs CHM6172 comparison, while 14 genes were significantly downregulated in the TND399 vs CND399 comparison.

Correlation analysis revealed that several key genes associated with in the flavonoid pathway showed strong positive correlations with specific metabolites in the resistant variety HM6172 (Figure 4-9B). In particular, *TaF3GT* (TraesCS2B02G040500) and *TaPAL* (TraesCS2D02G377200) showed significant positive correlations with rutin accumulation ($r = 0.83$, $P < 0.01$ and $r = 0.66$, $P < 0.01$, respectively). Similarly, *TaF5H* (TraesCS2A02G490100) and *TaF3'H* (TraesCS6B02G354500) were strongly correlated with chrysin levels ($r = 0.89$, $P < 0.01$ and $r = 0.82$, $P < 0.01$, respectively). In addition, a peroxidase gene (TraesCS1D02G130200) showed a significant positive correlation with luteolin ($r = 0.94$, $P < 0.01$), while *TaCHS* (TraesCS2A02G527700) was significantly correlated with luteolin-7-O-glucoside ($r = 0.66$, $P = 0.02$). In contrast, in the susceptible variety ND399, two DEGs (TraesCS5B02G209200 and TraesCS3D02G412700) showed significant negative correlations with catechin and glycitin accumulation ($r = -0.84$ and -0.86 , $P < 0.01$). These results suggest that these DEGs may play important roles in regulating flavonoid biosynthesis during aphid infestation.

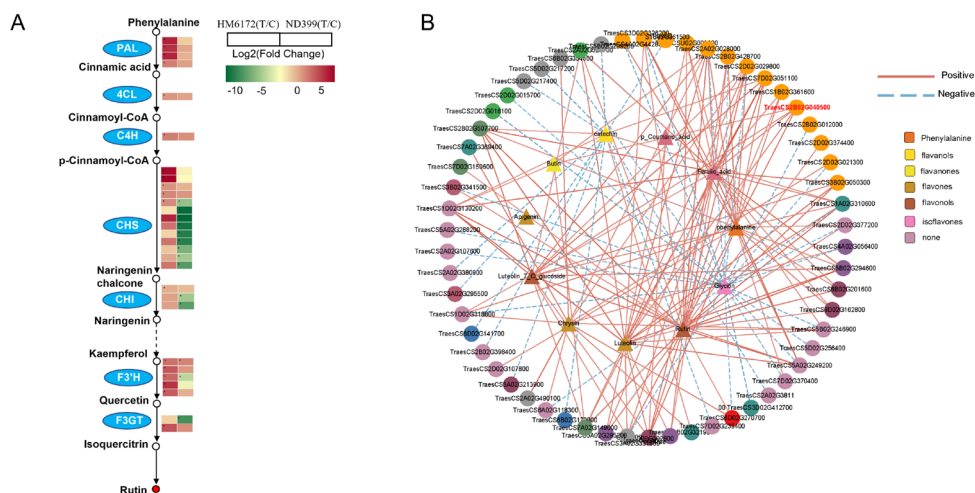


Figure 4-9: Combined transcriptome and metabolome analysis. (A) Expression profiles of genes involved in rutin biosynthesis pathway in cv. HM6172 and cv. ND399 in response to aphid infestation. Circles represent metabolites, ellipse represent genes ($*P < 0.05$). The heatmap scale ranges from -10 to 5 on a log2 fold change. (B) Correlation analysis of DEGs and differential flavonoid metabolites of wheat after aphid infestation. Triangles represent differential flavonoids and circles represent DEGs. Red solid lines indicate positive correlation, blue dashed lines indicate negative correlation, as determined by the absolute value of $|r| > 0.6$.

3.10. Functional characterization of *TaF3GT* in regulating rutin biosynthesis in wheat

TaF3GT was predicted to be positively correlated with rutin biosynthesis in aphid-resistant variety HM6172 as described above, its regulatory role was further investigated. Dynamic expression pattern analysis revealed that *TaF3GT* were significantly upregulated in cv. HM6172 at 12h after aphid feeding, and reached peak levels (283.03 ± 75.76 -fold) at 24 h post-infestation. However, no significant change in *TaF3GT* expression was observed in cv. ND399 following aphid infestation (Figure 4-10A). To further examine the role of *TaF3GT* in rutin biosynthesis, VIGS was employed to downregulate *TaF3GT* expression in the aphid-resistant variety HM6172. At 10 days post-inoculation, wheat leaves infiltrated with the BSMV: *TaPDS* recombinant vector showed severe photobleaching, confirming the effectiveness of the VIGS system in this study (Figure 4-10B). Gene expression analysis revealed that the transcript level of *TaF3GT* were significantly reduced to 0.027-fold of those in controls at 48 hours (Figure 4-10C). Correspondingly, the rutin content in *TaF3GT*-silenced leaves was significantly lower than in wild-type plants and those inoculated with the empty vector BSMV:00 (Figure 4-10D, $P < 0.05$).

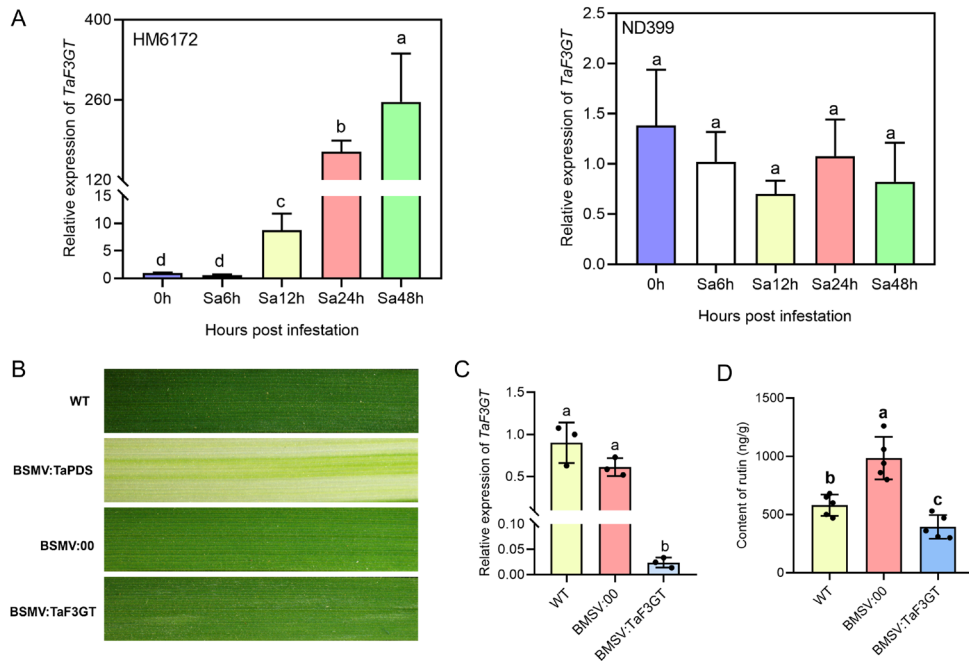


Figure 4-10: *TaF3GT* involved in regulating rutin biosynthesis in wheat. (A) The expression of *TaF3GT* in cv. HM6172 and cv. ND399 infested with *S. avenae* at 6h, 12 h, 24 h and 48h post-infestation. Three replicates were conducted for each treatment. Data are presented as mean $\pm$ standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$). (B) The phenotypes of wheat variety HM6172 were inoculated with barley stripe mosaic virus BSMV: 00 (Control) and recombinant BSMV: *TaPDS*, BSMV: *TaF3GT* at two-leaf stage, and photographed 10 days after inoculation. (C) Expression levels of *TaF3GT* in wheat leaves inoculated with BSMV: *TaF3GT* at 5 days ($n = 3$). Wide type (WT) and wheat leaves inoculated with BSMV: 00 were set as control groups. (D) Content of rutin in WT and wheat leaves inoculated with BSMV: 00 or BSMV: *TaF3GT* at 5 days ($n = 5$).

3.11. Transcriptional activity of AT-hook motif-containing protein *TaAHL19* on *TaF3GT* expression

A previous study demonstrated that the AT-hook motif nuclear-localized protein 20 bind to the F3GT promoter and regulated rutin biosynthesis in buckwheat (Zhang et al., 2023). In the present study, we found that the expression of a AT-hook-containing transcription factor *TaAHL19* was significantly upregulated in the aphid-resistant wheat cv. HM6172 after 24 h and 48 h of aphid feeding, whereas no significant changes were observed in the susceptible cv. ND399 (Figure 4-11A). Sequence analysis identified five potential AT-hook binding sites in the *TaF3GT* promoter region (Figure 4-11B). This finding suggested that *TaAHL19* may also bind to this promoter and regulate *TaF3GT* expression. The full-length coding sequence of *TaAHL19* is 771 bp, encoding a 257-amino-acid protein with a predicted molecular

weight of 23.86 kDa. Phylogenetic analysis revealed that TaAHL19 is most closely related to TaAHL29 and shares high similarity with *Arabidopsis* homologs AtAHL15, AtAHL19, and AtAHL20 (Figure 4-11C).

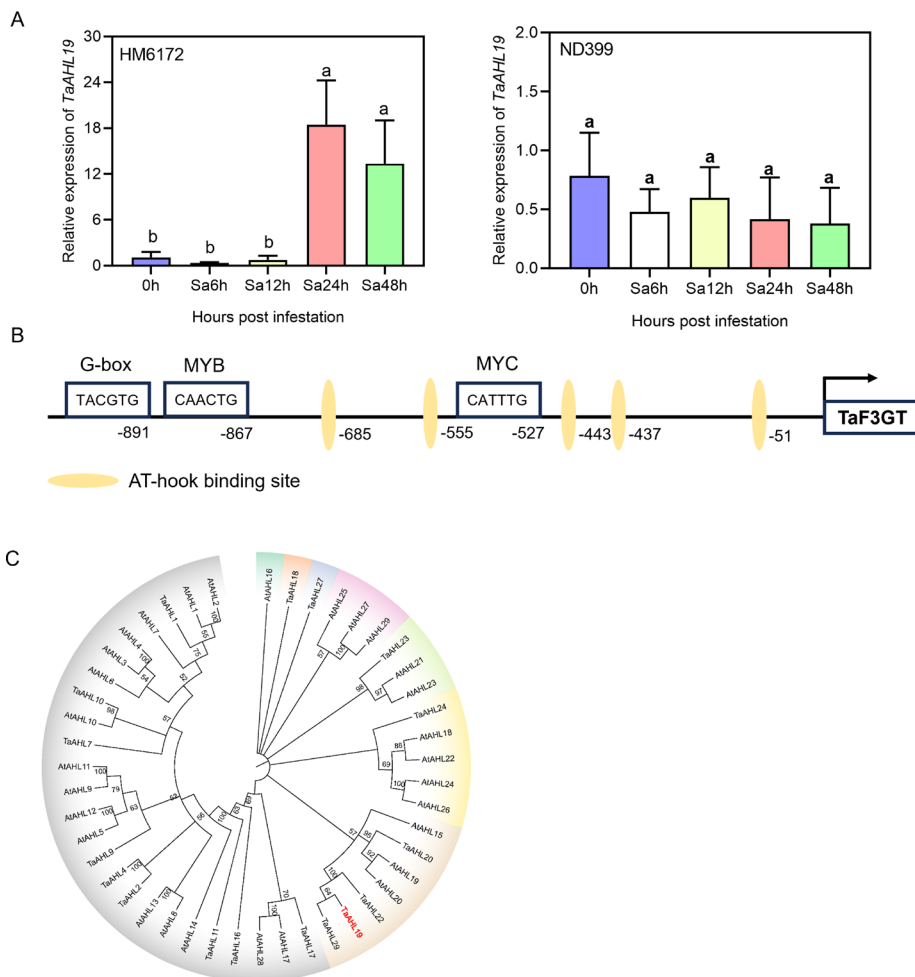


Figure 4-11: Functional characterization of *TaAHL19*. (A) The expression of *TaAHL19* in cv. HM6172 and cv. ND399 infested with *S. avenae* at 6h, 12 h, 24 h and 48h post-infestation. Three replicates were conducted for each treatment. Data are presented as mean $\pm$ standard error. (B) Schematic diagrams of the *TaF3GT* promoter. AT-hook binding site were denoted using yellow ellipse. (C) Phylogenetic tree constructed by comparing the amino acid sequences of *TaAHL19* proteins from wheat and *Arabidopsis thaliana*. 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.

To investigate the transcriptional activity of *TaAHL19* in regulating *TaF3GT* gene expression, we performed Y1H and Dual-luciferase assays. In the Y1H assay, positive control and yeast strains co-transformed with AD-*TaAHL19* and pHIS2-*proTaF3GT*

were able to grow on SD/-Trp/-His/-Leu medium supplemented with 50 mM 3-AT, while the strain carrying the empty AD vector with pHIS2-*proTaF3GT* failed to grow (Figure 4-12A), indicating that *TaF3GT* is transcriptionally regulated by *TaAHL19*. Dual-luciferase assay was then performed to confirm the binding of *TaAHL19* to the *TaF3GT* promoter. The promoter sequence of *TaF3GT* was fused with the LUC reporter gene to construct the *proTaF3GT*-LUC vector, and the *TaAHL19* CDS was cloned into pGreenII62-SK to generate the effector plasmid. The dual-luciferase assays in *N. benthamiana* leaves showed that co-infiltration with pGreenII62-*TaAHL19*-SK and pGreenII0800-*proTaF3GT*-LUC led to stronger luminescence signals compared to the control (Figure 4-12B). The relative LUC/REN ratio was significantly increased by approximately 13.91-fold in *N. benthamiana* leaves infiltrated with pGreenII0800-*proTaF3GT*-LUC and pGreenII62-*TaAHL19*-SK than that of control plants (Figure 4-12C), suggesting that *TaAHL19* specifically binds to the *TaF3GT* promoter *in vivo*.

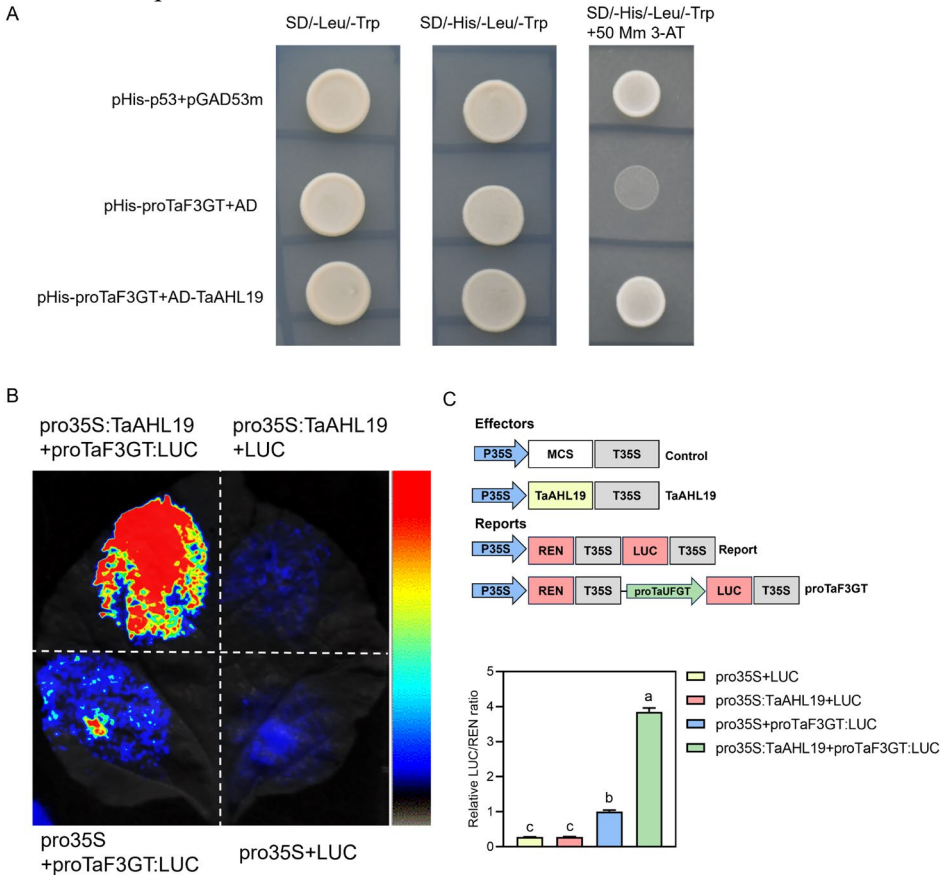


Figure 4-12: *TaAHL19* binds to the promoter region of *TaF3GT*. (A) Yeast one hybrid experiment of *TaAHL19* and *TaF3GT* promoter. (B) Dual-luciferase reporter assays detected the binding activity of *TaAHL19* in the promoter of *TaF3GT*. The relative luciferase activities

in different samples were calculated by normalizing the LUC values against REN ($n = 6$). Data are presented as mean $\pm$ standard error. Different lowercase letters represent significant differences among different groups ($P \leq 0.05$).

3.12. TaAHL19 overexpression enhanced aphid resistance by promoting rutin biosynthesis in wheat

To further investigate the role of *TaAHL19* in rutin biosynthesis and wheat resistance against aphids, we generated transgenic wheat lines overexpressing *TaAHL19*. Two independent T3 generation of transgenic lines, namely *TaAHL19*-OE2 and *TaAHL19*-OE6 were obtained (Figure 4-13A), and RT-qPCR analysis confirmed that the expression levels of *TaAHL19* in the transgenic lines OE2 and OE6 were approximately 37.23-fold and 11.07-fold higher than in WT plants, respectively (Figure 4-13B). Consequently, the transcript levels of the target gene *TaF3GT* were also significantly upregulated, reaching about 43.61-fold of the WT level (Figure 4-13B). Subsequent LC-MS/MS analysis revealed that the content of rutin in *TaAHL19*-OE2 plants (230.00 ± 28.87 ng/g) was significantly higher than that in WT leaves (122.50 ± 16.52 ng/g) (Figure 4-13C). Aphid bioassays revealed that the number of nymphs produced per adult of *S. avenae* was significantly lower on *TaAHL19*-OE plants than on the WT (Figure 4-13D). Notably, the overexpression of *TaAHL19* had no negative effects on key agronomic traits of wheat (Figure 4-13E), as no significant differences were observed between transgenic and WT plants in terms of grain length, grain width, and thousand-grain weight (Figure 4-13F). These results demonstrate that *TaAHL19* enhanced wheat resistance to aphids by positively regulating rutin biosynthesis.

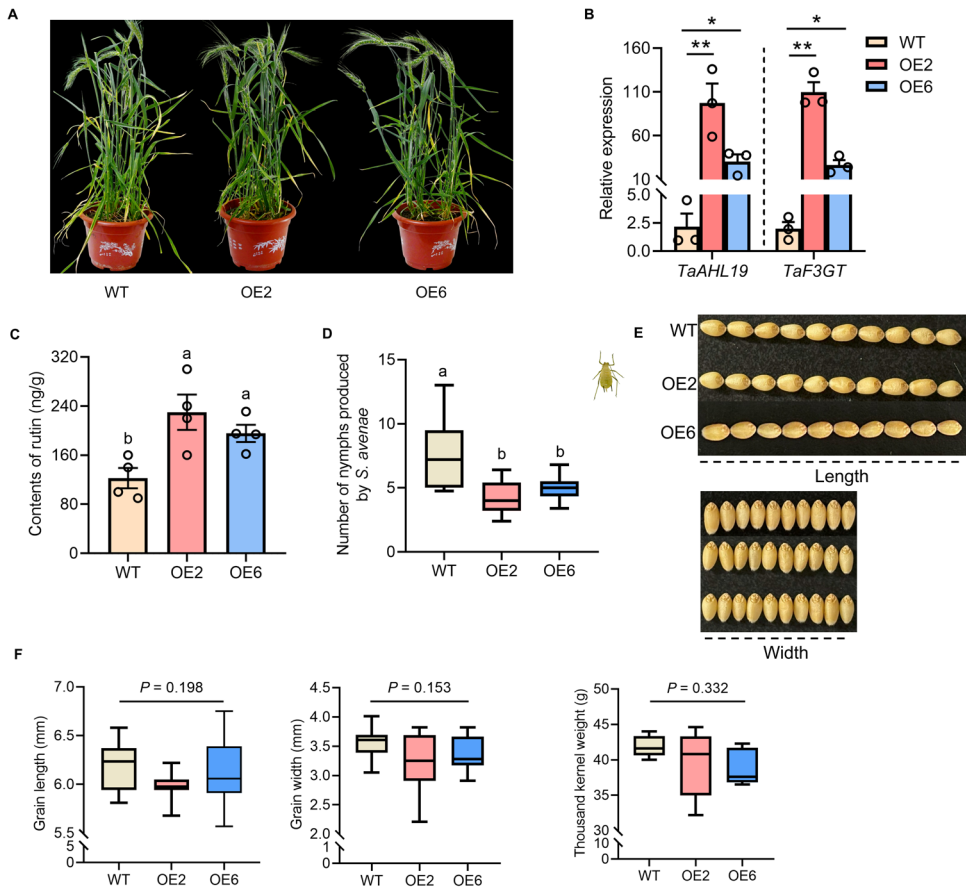


Figure 4-13: *TaAHL19* overexpression enhanced aphid resistance. (A) Plant phenotype of *TaAHL19*-overexpressing lines (OE2 and OE6) and WT Fielder line. (B) The expression levels of *TaAHL19* and *TaF3GT* in *TaAHL19*-overexpressing lines (OE2 and OE6) and WT line (n = 3). (C) Content of rutin in *TaAHL19*-overexpressing line OE2, OE6 and WT line. (n = 4) (D) Number of nymphs produced by each aphid in 5 days after feeding on *TaAHL19*-overexpressing lines (OE2 and OE6) and WT line (n = 18). (E) Grains of *TaAHL19*-overexpressing lines (OE2 and OE6) and WT Fielder line. (F) Grain length, grain width and thousand-grain weight of *TaAHL19*-overexpressing lines (OE2 and OE6) and WT lines (n = 10).

3.13. JA signaling promotes *TaAHL19* and *TaF3GT* expression

To elucidate the potential relationships between JA signaling pathway with flavonoid biosynthesis pathway, wheat leaves were exogenously treated with 0.2 mM, and 0.5 mM JA for 12 h. RT-qPCR analysis revealed that expression levels of *TaAOS* and *TaFAD*, JA pathway-associated genes, were significantly upregulated after JA treatment (Figure 4-14A, $P < 0.05$). In addition, we found that 0.5 mM JA significantly induced both *TaAHL19* and *TaF3GT* expression in wheat seedlings (Figure 4-14B, $P < 0.05$). Seedlings sprayed with 0.5 mM JA exhibited an

approximately 59-fold increase compared to untreated controls, suggesting a potential association between JA signaling and the regulation of flavonoid biosynthesis.

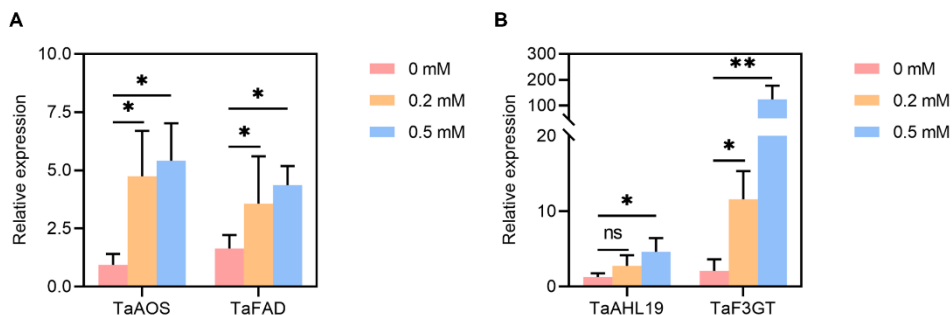


Figure 4-14: Exogenous JA treatment induces *TaAHL19* and *TaF3GT* expression in wheat. (A) Relative expression levels of the JA biosynthesis-related genes *TaAOS* and *TaFAD* after treatment with 0, 0.2, or 0.5 mM JA for 12 h. (B) Relative expression levels of *TaAHL19* and *TaF3GT* after exogenous JA treatment. Data are presented as means $\pm$ SD ($n = 3$). Asterisks indicate significant differences between treatments (* $P < 0.05$, ** $P < 0.01$); ns, not significant.

4. Discussion

Consistent with a previous study (Liu et al., 2024), age-stage life table in our study confirmed that the wheat variety HM6172 exhibited resistance against aphid. Further analysis of aphid feeding behavior revealed that on cv. HM6172, *S. avenae* required significantly more time to initiate the first phloem ingestion and spent less time feeding from the phloem. A shortened duration of phloem feeding by insects on host plants is a well-established indicator of antibiotic resistance against phloem-feeding pests (Calderon and Backus, 1992; Kloth et al., 2021). This pattern aligns with observations on the resistant wheat line Ww2730, where aphids also exhibited significantly reduced phloem feeding duration (Hu et al., 2008). Taken together with the reduced aphid performance observed on cv. HM6172, our results demonstrated that this variety possesses antibiotic resistance to wheat aphids. In addition to its pest resistance, cv. HM6172 has been documented to confer immunity against wheat stem rust *Puccinia graminis* (Sun et al., 2004). Furthermore, this variety exhibits excellent adaptability and has been widely cultivated in the major wheat-producing regions of China's Yellow-Huai-Hai River Valleys, including Hebei, Shandong, and Henan provinces (Liu et al., 2007; Ma et al., 2002). Thus, it is worthwhile to understand the mechanisms by which this variety balances multiple resistances with agronomic performance.

While several studies have characterized the transcriptomic responses of wheat to insect attack under controlled conditions (Wang et al., 2022b), this study investigated the transcriptomic and metabolomic responses of wheat to aphid infestation in field conditions. This approach was chosen to better capture the induced defense responses of wheat under natural environmental conditions. Under field conditions, environmental factors such as temperature, light, humidity, and other biotic stresses could not be fully controlled. Some of the observed differences between cv. HM6172

and cv. ND399 may have been influenced by environmental variation in addition to aphid infestation and genotype-specific responses. The use of net enclosures may also have modified the local microenvironment and excluded natural enemies. However, cv. HM6172 and cv. ND399 were grown and evaluated under the same field conditions and subjected to the same experimental procedures, minimizing the potential influence of these factors on the comparison between the two cultivars. Importantly, the key findings obtained from the field experiments were subsequently supported by functional validation under controlled conditions. Although field conditions are complex and variable, and physiological changes in wheat could be influenced by multiple biotic and abiotic stresses, we believed that our research findings reflect the real induced defense responses of wheat to aphid feeding in the natural environment. And, transcriptomic and metabolomic analyses revealed a significant upregulation of numerous defense-related genes and metabolites, demonstrating the initiation of a robust defense program in wheat following aphid infestation in the field. Previous studies also have characterized defense responses in commercial maize to *O. furnacalis* and in wheat to *S. mosellana* using transcriptomic and metabolomic profiling under field conditions (Guo et al., 2023; Wang et al., 2022b).

According to transcriptome analysis, approximately 4,000 differentially expressed genes were identified in either cv. HM6172 or cv. ND399 following aphid feeding, indicating a strong physiological response in wheat to aphid infestation. In the comparison of THM6172 vs. CHM6172, the up-regulated DEGs were significantly enriched in pathways related to phenylpropanoid, flavonoid, diterpenoid, and steroid biosynthesis, which are known to be involved in biotic stress responses of plants (Dong and Lin, 2021). However, the up-regulated pathways in TND399 vs. CND399 were primarily associated with primary metabolic processes, such as amino sugar and nucleotide sugar metabolism, and cyanoamino acid metabolism. Compared to TND399 vs. CND399, phenylpropanoid and flavonoid biosynthesis were the only significantly up-regulated pathways in THM6172 vs. CHM6172. This finding is consistent with a previous study in which flavonoid biosynthesis was specifically enriched in a resistant wheat variety infested by *S. mosellana*, highlighting their important role in wheat resistance to insect pests (Wang et al., 2022b). The flavonoid biosynthetic pathway represents a major downstream branch of phenylpropanoid metabolism (Buer et al., 2010). In plants, flavonoids are synthesized from phenylalanine, including the subgroups of flavanones, flavones, isoflavones, flavanols, flavonols, and anthocyanidin (Shen et al., 2022). Several studies have demonstrated that flavonoid compounds contribute to plant resistance to pests and pathogens (Górniak et al., 2019; Tayal et al., 2020; Onkokesung et al., 2014; Martens and Mithofer, 2005). In the present study, the expression levels of key phenylpropanoid and flavonoid biosynthesis-related genes including *TaPAL*, *Ta4CL*, *TaDFR*, *TaCHS*, and *TaF3'H* were significantly upregulated in the resistant wheat variety HM6172 following aphid feeding, whereas no significant changes were observed in the susceptible variety ND399. Previous studies showed the transcript levels of *Ta4CL* and *TaF3'H* were elevated in resistant wheat lines infected with powdery mildew

Blumeria graminis (Xin et al., 2012), and the expression levels of *CHS* and *PAL* were upregulated when tea plants infected with gray blight (Wang et al., 2021). Upon herbivore attack, resistant wheat variety LX99 exhibited higher expression levels of *TaPAL* and *Ta4CL* following infestation by the orange wheat blossom midge, *Sitodiplosis mosellana* (Wang et al., 2022b). In addition, overexpression of the flavonoid biosynthetic gene *CoDFR* increased flavonoid content and enhanced oil tea (*Camellia oleifera*) resistance to anthracnose (Yang et al., 2022), which suggested flavonoid biosynthesis plays a key role in plant resistance to pests and pathogens.

Consistent with these transcriptional changes, metabolomic analysis in this study revealed that several flavonoids such as rutin, luteolin, chrysin, and luteolin-7-O-glucoside were specifically induced in the resistant wheat variety upon aphid infestation. In cv. HM6172, the contents of rutin and luteolin increased by approximately 17.12-fold and 5.22-fold, respectively, after aphid feeding, whereas these compounds were undetectable in cv. ND399. These results suggested that the induced accumulation of these specific flavonoids may contribute to aphid resistance in wheat. Rutin, a glycosylated flavonoid with antioxidant and anti-inflammatory properties, is notably abundant in Tartary buckwheat *Fagopyrum tataricum* and has been documented to contribute to plant resistance against both pathogens and insects (Pandey et al., 2012; Kim et al., 2013; Lu et al., 2016; Yang et al., 2016). Exogenous application of rutin has been shown to enhance resistance in rice against the bacterial wilt pathogen *Ralstonia solanacearum* and the leaf spot pathogen *Pseudomonas syringae*, as well as in tomato against *Xanthomonas perforans* (Yang et al., 2016; Farahani and Taghavi, 2018). Overexpression of the key gene *FtCYP94C1* involved in the flavonoid metabolism pathway increased the content of the flavonoid compound rutin in the tartary buckwheat *F. tataricum* and subsequently improved plant resistance to the *Fusarium* wilt disease *Rhizoctonia solani* (He et al., 2023). Dietary supplementation with purified rutin significantly increased larval mortality and reduced the body weight of *Trichoplusia ni* (Hoffmann-Campo et al., 2001). In the present study, direct feeding on an artificial diet containing rutin adversely affected the fecundity of both *S. avenae* and *S. graminum*, which determine the roles of rutin in conferring cereal aphid resistance in wheat. Interestingly, rutin exposure also markedly reduced the fecundity and survival of the polyphagous insects *M. persicae* and *S. frugiperda*, suggesting that rutin may confer broad-spectrum resistance against herbivorous insects. It is hypothesized that rutin impairs insect performance by interacting with arginine kinase, a key enzyme involved in cellular energy metabolism in insects (Wu et al., 2009).

Biosynthesis of flavonoid glycosides are catalyzed by UDP-dependent glucosyltransferases (UGTs) that are widely present in plants. Previous studies have established that rutin biosynthesis involves UDP-dependent glucosyltransferases (UGTs), and the regulation of UGT expression significantly influences rutin accumulation (Zhao et al., 2024). Flavonol 3-O-glucosyltransferase (F3GT) and flavonol 3-O-glucoside rhamnosyltransferase (3G6RhaT) are key enzymes in the final step of rutin biosynthesis, a pathway well-established in Tartary buckwheat (Yao et al., 2021). In this study, integrated transcriptome and metabolome analyses revealed

that the accumulation of flavonoids including rutin, chrysin, luteolin, and luteolin-7-O-glucoside in the aphid-resistant variety HM6172 was positively regulated by the genes *TaPAL*, *TaCHS*, *TaF3H*, *TaF3'H*, and *TaF3GT*. Furthermore, aphid feeding specifically induced the expression of *TaF3GT* in cv. HM6172. Although F3GT has been reported to positively regulate flavonol synthesis and accumulation in buckwheat (Zhu et al., 2024), but roles of *TaF3GT* involved in rutin biosynthesis has not been reported in wheat to date. In this study, we employed BSMV-mediated gene silencing to investigate the potential role of *TaF3GT* in rutin biosynthesis. The results showed a significant reduction in *TaF3GT* expression in BSMV: *TaF3GT*-treated wheat leaves, confirming the effectiveness of BSMV-induced silencing for functional gene studies in wheat. Consequently, the silenced plants exhibited decreased rutin levels in their leaves, demonstrating that *TaF3GT* promotes rutin biosynthesis in wheat.

Transcription factors such as MYB and bHLH families play pivotal roles in regulating flavonoid synthesis by modulating the expression of key enzymes involved in the flavonoid biosynthetic pathway (LaFountain and Yuan, 2021). *SoMYB44* interacted with *SobHLH130* to trigger *SoUFGT1* expression in lilac *Syringaoblata*, which contributes to the anthocyanin accumulation (Wang et al., 2025). *FtMYB16* directly repressed the expression of *FtMATE1* gene encoding the transporter of isoquercitrin for rutin accumulation in tartary buckwheat (Li et al., 2019). Overexpression of *CtMYB76* in tobacco significantly upregulated the expression levels of *NtFLS*, *NtF3'H*, *NtF3H*, *NtCHI*, and *NtCHS*, and concomitantly led to a marked accumulation of quercetin and kaempferol (Tan et al., 2025). AT-hook motif nuclear-localized (AHL) is an important transcription factor that regulates diverse biological processes including plant growth, primary and secondary metabolism, plant pathogens response, and hormonal homeostasis (Zhang et al., 2022f). Several AHL members are involved in regulating plant immunity; for instance, overexpression of AHL15, AHL20, and AHL27 suppressed flg22-induced expression of the immunity-related gene FRK1 in protoplasts (Lu et al., 2010). *AtMYB12* was shown to regulate rutin accumulation and inhibit insect growth, thereby improving plant resistance against *Spodoptera litura* and *Helicoverpa armigera* (Misra et al., 2010; Pandey et al., 2012). In this study, we observed that aphid infestation significantly induced the expression of *TaAHL19* in the resistant wheat line HM6172, but not in the susceptible line cv. ND399. Its expression pattern closely mirrored that of *TaF3GT*, a key gene in rutin biosynthesis. Transient expression assays in *N. benthamiana* leaves demonstrated that *TaAHL19* enhances luciferase expression driven by the *TaF3GT* promoter, confirming that *TaAHL19* directly activated *TaF3GT* expression *in vivo*. Based on these findings, we proposed that *TaAHL19* plays a critical role in regulating rutin biosynthesis in wheat. However, a gene *Fh06G015130* of tartary buckwheat, encoding AT-hook motif nuclear-localized protein 20, was found to bind to the promoters of *F3GT* and negatively regulate its expression and rutin biosynthesis (Zhang et al., 2023). These findings indicated that AHL transcription factors can play divergent roles in regulating rutin biosynthesis across different plant species.

Phytohormones such as SA and JA are important signaling molecules involved in inducing plant immunity and conferring plant resistance to aphid attack (Shih et al.,

2022). Aphid feeding elicits SA- and JA-dependent plant defense responses, and JA-mediated defenses have been demonstrated to be more effective against aphids (Züst and Agrawal, 2016). For example, the exogenous application of JA to tomato plants significantly negatively affected aphid population growth (Cooper et al., 2004). Our results also showed that several JA-related genes, including *TaLOX*, *TaAOS*, *TaOPR*, *TaPKT3*, and *TaJMT*, were significantly upregulated only in the resistant wheat variety HM6172. What is more, aphid infestation triggered a stronger JA response in the resistant wheat variety HM6172 than in the susceptible variety ND399. JA induces the activation of transcription factors such as MYB, ERF, WRKY, and bHLH, thereby modulating the biosynthesis of flavonoids (Zhou and Memelink, 2016). A previous study showed exogenous application of methyl jasmonate (MeJA) can induce the expression of *OsF3H* and *OsCHI* in the flavonoid biosynthesis pathway, as well as the accumulation of flavonoids (Chen et al., 2025). In this study, treatment with JA led to a significant upregulation in the expression of *TaAHL19* and *TaF3GT*, two genes associated with rutin biosynthesis. The JA-induced accumulation of *FtBPM3* mediated the degradation of FtERF-EAR3, thereby enhancing *FtF3H* expression and promoting subsequent rutin biosynthesis (Ding et al., 2022). It is proposed that JA is involved in flavonoid biosynthesis in HM6172, such as rutin biosynthesis induced by aphid feeding, thereby enhancing wheat resistance to aphids.

Transgenic wheat lines overexpressing *TaAHL19* were generated to investigate its role in the regulation of rutin accumulation and plant resistance. RNA-seq and KEGG analysis revealed that the upregulated DEGs in these lines were significantly enriched in the flavonoid biosynthesis pathway. Consistently, *TaAHL19* overexpression elevated the expression of *TaF3GT* and increased rutin content, confirming that *TaAHL19* positively regulates rutin biosynthesis by promoting *TaF3GT* expression. When challenged with *S. avenae*, the fecundity was significantly reduced on *TaAHL19*-overexpressing plants compared to WT plants, indicating that *TaAHL19*-induced rutin accumulation enhances aphid resistance. Given high rutin content in plants is known to enhance resistance to various pathogen diseases (Farahani and Taghavi, 2018; Joshi et al., 2020) and adaptation to high-altitude environments (Gao et al., 2025), genetic engineering of key genes in the rutin biosynthesis pathway represents a promising strategy for improving crop resistance to both biotic and abiotic stresses.

An important consideration for the application of plant resistance mechanisms in crop improvement is the potential trade-off between defense and growth. Activation of defense signaling and the production of specialized metabolites require metabolic resources and may therefore impose fitness or yield costs, particularly when defense responses are constitutively activated. In this study, overexpression of *TaAHL19* enhanced wheat resistance to aphids and increased rutin accumulation. Notably, this enhanced resistance showed no adversely effects on yield, as no significant differences in grain length, width, or thousand-grain weight were observed between transgenic and WT lines, suggesting that enhanced resistance mediated by *TaAHL19* did not cause an obvious yield penalty under the conditions tested. Nevertheless, more comprehensive evaluation of yield-related traits across multiple growing seasons and

environments will be required to determine whether enhanced defense affects plant growth and productivity under diverse agricultural conditions.

Additionally, we found that the expression levels of *TaF3GT* and *TaAHL19* were significantly upregulated only in the aphid-resistant wheat variety following aphid infestation, while no notable changes were observed in the aphid-susceptible variety. Variations in the coding sequence and promoter region of resistance genes of plant could affect the binding activity of upstream transcription factors, thereby influencing transcriptional regulation and the level of plant resistance against abiotic stress, pests and pathogens (Gan et al., 2025; Guo et al., 2025). For instance, CTB5, a homeodomain-leucine zipper (HD-Zip) transcription factor, confers enhanced cold tolerance through four natural variations in the promoter and coding regions (Guo et al., 2025). Promoter-localized single-nucleotide polymorphisms (SNPs) in the *GmCML* were significantly associated with plant resistance, and haplotypes 2 and haplotypes 3 conferred enhanced resistance against *Fusarium oxysporum* (Zhou et al., 2025a). However, comparison of the coding sequence and promoter regions of *TaF3GT* and *TaAHL19* between resistant and susceptible varieties revealed no sequence variations. The distinct expression patterns of these two genes in resistant and susceptible varieties, and the molecular mechanisms by which these patterns confer differing resistance levels remain to be further elucidated.

In summary, comparing the induced responses of resistant (cv. HM6172) and susceptible (cv. ND399) varieties to aphid infestation through multi-omics revealed the critical role of induced flavonoid rutin in conferring wheat resistance against aphids. Functional validation firstly confirmed that the transcription factor *TaAHL19* confers aphid resistance by binding to the *TaF3GT* promoter, thereby enhancing rutin biosynthesis in wheat. These results provided profound insights into the molecular mechanisms of aphid resistance in wheat and establish a theoretical foundation for breeding resistant wheat germplasm.

5. Supplementary file

Table S4-1. All AHL amino acid sequences in wheat and *Arabidopsis thaliana*

>TaAHL1

MEAKPGEASSPTATAAAAAAPVAAAAAAVVPEATVSFQSPASVPPPTASHAPAPSPVE
MVSSSGLFVPPGMMTAMVAAAGRGALSAGPLVKVGKKRGRPRKYGPDGSLIQPLN
ATPISASAPMSAAVAAGQYTPAAAVGAAMKRGRGRPLDFAAAAAKPYHHQLHQPP
QQQFGFHFSSIGDMVACSAGGNFTPHIITVAPGEDVTMKVISFSQQGPRAICILSANG
VISNVTLRQPDSSGGTLTYEGRFELLSLSGSFMPTENSGARSRSGGMSVSLASPDGRV
VGGGVAGLLVAASPVQIVVGSFLPSYPMEPKQKKARVSAAPLSQAPPAVQLSSAD
THSSEQGQHSSAAPRMNVVTSSAYSADQNWASTVVQSAPAEASRSTSSGDLNLTAS
GS

>TaAHL2

MEGGDMSEAAGEAVYGEDMPKSPLHPQPQPIIGAYLDDAHEGSASYSAPRSSFPAA
GNGVVHVPAMSASSEPPKQKRGRPRKYAPDGTKPPAIFPAPYPIGVVASPTPALPPGF
TLGLWGLGVVRPQAPPALPSLPPPPSENSKQRAKKKRVWPPSSTTSKKQRELAVAE
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>TaAHL7

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>TaAHL9

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>TaAHL10

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>TaAHL11

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>TaAHL16

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>TaAHL18

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>TaAHL20

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>TaAHL22

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>TaAHL23

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>TaAHL24

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>TaAHL25

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>TaAHL27

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>TaAHL29

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>AtAHL1

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>AtAHL2

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>AtAHL3

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>AtAHL4

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>AtAHL5

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>AtAHL6

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>AtAHL7

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>AtAHL8

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>AtAHL9

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>AtAHL10

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>AtAHL11

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>AtAHL12

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>AtAHL13

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>AtAHL14

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>AtAHL15

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>AtAHL16

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>AtAHL17

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>AtAHL18

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>AtAHL19

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>AtAHL20

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>AtAHL21

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>AtAHL22

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>AtAHL23

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>AtAHL24

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GSVVGPLL CAGPVVMAASF SNAAYERLPLEEDEM QTPVHGGGGGGSLESPPMMG
QQLQHQQQAMSGHQGLPPNLLGSVQLQQQHDQSYWSTGRPPY

>AtAHL25

MSSYMHPLLQELHLQRPEDSRTPPDQNNMELNRSEADEAKAETTPTGGATSSATA
SGSSSGRRPRGRPAGSKNKPPTIITRDSNVLRSHVLEVTSGSDISEAVSTYATR RG
CGVCIISGTGAVTNVTIRQPAAPAGGGVITLHGRFDILSLTGTALPPPAPPAGGLTV
YLAGGQGQVVG GNVAGSLIASGPVVLMAASFANAVYDRLPIEEEETPPRRTTG VQQ
QQPEASQSSEVTGSGAQACESNLQGGNGGGGVAFYNLGMNMNNFQFSGGDIYGMS
GGSGGGGGGATRPAF

>AtAHL26

MDPVQSHGSQSSLPPPFHARDFQLHLQQQQQHQQQHQQQQQQQFFLHHHQQPQRN
LDQDHEQQGGSILNRSIKMDREETSDNMDNIANTNSGSEKEMSLHGGEGGSGGGG
SGEQMTRRPRGRPAGSKNKPAPIIITRDSANALRTHVMEIGDGC DIVDCMATFARR
RQRGVCVMSGTGSVTNV TIRQPGSPPGSVVSLHGRFEILSLSGSFLPPPAPPAATGLS
VYLAGGQGQVVG GSVVGPLLCSGPVVMAASF SNAAYERLPLEEDEM QTPVQGGG
GGGGGGGGMGSPMMGQQQAMAAMAAAQGLPPNLLGSVQLPPPQNDQQYWST
GRPPY

>AtAHL27

MEGGYEQGGGASRYFHNLF RPEIHHQQLQPQGGINLIDQH HHQHQQHQQQQQPSD
DSRESHNSKDH HQGRPDSDPNTSSSAPGKRPRGRPPGSKNKA KPPIIVTRDSPNAL
RSHVLEVSPGADIVESVSTYARRRGRGVSVLGGNGTVSNVTLRQPVTPGNGGGVSG
GGGVVTLHGRFEILSLTGTVLPPPAPPAGGLSIFLAGGQGQVVG GSVVAPLIASAPV
ILMAASF SNAVFERLPIEEEEEGGGGGGGGGGGPPQMQQAPSASPPSGVTGQQQLG
GNVGGYGFGSDPHLLGWGAGTPSRPPF

>AtAHL28

METVGRPRGRPRGSKNKPAPIFVTIDPPMSPYILEVPSGNDVVEALNRF CRGKAIGF
CVLSGSGSVADVTLRQPSAAPGSTITFHGKFDLLSVSATFLPPLPPTSLSPVSNFFTV
SLAGPQGVIGGFVAGPLVAAGTVYFVATSFKNPSYHRLPATEEEQRNSAEGEEEGQ
SPPVSGGGGESMYVGGSDVIWDPNAKAPSPY

>AtAHL29

MDGGYDQSGGASRYFHNLF RPELHHQLQPQPQLHPLPQPQPQPQQNSDDES DS
NKDPGSDPVTSGSTGKRPRGRPPGSKNKP PPVIVTRDSPNVLRSHVLEVSSGADIVE
SVTTYARRRGRGV SILSGNGTVANVSLRQPATTA AHGANGGTGGVVALHGRFEILS
LTGTVLPPPAPP GSGGLSIFLSGVQGGQVIGGNVAPLVASGPVILMAASF SNAFERL
PLEDEGGEGGEGGEVGE GGGGGGPPATSSSPPSGAGQGQLRGNMSGYDQFAGDP
HLLGWGAAAAAAPP RPAF

>TaAHL1

MEAKPGEASSPTATAAAAA PVAAAAAVVPEATVSFQSPASVPPPTASHAPAPSPVE
MVSSSGLFVPPGMMTAMVAAA GRGALSAGPLVKVGKKRGRPRKY GPDGSLIQPLN
ATPISASAPMSAAVAAGQYTPAAAVGAAMKRGRGRPLDFAAAA KPYHHQLHQPP
QQQFGFHFS SIGDMVACSAGGNFTPHIITVAPGEDVTMKVISFSQQGPRAICILSANG
VISNVTLRQPDSSGGTLTYEGRFELLSLSGSFMP TENS GARSRSGGMSVSLASPDGRV
VGGGVAGLLVAASPVQIVVGSFLPSYPMEPKQKKARVSAAPPLSQAPPAVQLSSAD

THSSEQGQHSSAAPRMNVVTSSAYSADQNWASTVVQSAPAEASRSTSSGDLNLTAS
GS

>TaAHL2

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TLGLWGLGVVRPQAPPALPSPLPPPPSENSKQRAKKKRVWPPSSTTSKKQRELAVAE
PGATGFAPQVITIQQGEDVAAKVMSYCGNGWAVFILSAEGAVRNVTLKQPASSRGT
VIYEGYFDIVCLSGVYLLSKSNGLSTLKGGSISLVGHDGCLFGGGLAGPLIAASPVQ
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ATGPTAAPNESSSGPGSPSN

>TaAHL4

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YLPSTKTDGVSSLKGGFAISLVGADGRIFGGGLAGPLIAASPVQVVIGRFAAGEKEETK
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>TaAHL7

MEGRVGIAVAGGHEAGLGLFRADVSMAPQGAACKVEYRSPSPSPSTPTSPPPQASA
GHGGGYAAATPHAWSFGGEQEKPEASAGDKGNGNGMQMAGDGEQVAGLSSGR
RRGRPRGSGRRQILATLGEWYALSAGGSFTPHVIIVPAREDVAARIMSFSQGRPRVC
ILAASGTISNVAFNKPGSSGSSDSTFTYEGLFEILQLTGSFTMAEEGGRKRTGGLSVSL
AGPDGRVIGGVVAGMLRAATPIQVIVGSFLPNLSLKQHQRMMGLHPQPSAVPALPVA
DAPPPVLTAAAMPISQAAPSNRHHGPPVPMAPLQVHANVEHTATTGPMNLNSSSAGF
TMVGWVPVGAQPMGHRLSPDINLCLTPQE

>TaAHL9

MDSTDDSSSLAASSGAASSSPYYVHHRPSPSPSTATQAPAFSPPPPPDAYPRHHHP
HPLDVFSFPMGPDPHHPGIPHPQMGPVSAAADDLGRKKRGRPRKYKPDGSALAPSP
APAPSPCTAIVPVTGSGGGPSSEKRRGRPPGSGKMQLASLGKSFLGTVTGTGFTPH
VIIPSGEDVAARIMSFSQQGPRAVCIMSASGAVSTATLHQDAGSGSVVKYEGRFEIL
CLSGSYLVIDDGVTTRNGGLCIALCGADHRVIGGSVGGVLTAAAGTVQVIVGSFMY
AGSKKSRKGKAGQESAAEEEEQTGDANGGRGGDGVGVGGEEEEEGPSLMPMPR
GEEELPSDAMMSGWTGMMRQMESRSCGGIDINSIRE

>TaAHL10

MEVRSEQLMAGRDLFGLPKSQPAPAPAAPGAPPSSAAMQSVRMAYTADGTPVFA
PVSSAVAPPGYQPAGSAAPPHGSSMSAARAAGNGVAAPPGMGEPsAKKKRGRPR
KYGPDAAMSLALVTVPATAAGSPAVTQGASGRPFSPTLPGNFVPSASPDGGKKRGRP
KGSTNKPRVDGAGPAGVGFTPHVLTQAGEDVSSKIMSFSQNGTRAVCVLSANGSI
SNVTLRQTGTSGGTVTYEGRFEILSLSGSIFVTDNGGQRTRTGGLSVSLAGPDGRLLG
GGVAGLLIAASPIQIVVGSFNAGGKKEPKQAPSEPVPLKVVVSTGMAANSPPSRGTL
SESSGGTASPRHQGYASTNNNQPPILSSMPWK

>TaAHL11

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GPSPIVWAMRTRVAPPPRLAEPIRPVYPPPPPPRAPLRTPTKEKRRRGRPRKVACLPPP
PGLGPPPGFAPSPAARNPAAHGGQDGLGGLQPHVLTIAVGEDIISRVMASVQLNSKAI
CILSALGVSREAVLVQPSGVILTHKGPLEIIRLSGSILTSNDHRSLRITLASVNSFVISGII
AGPLIAETPVQAILGSFHNSAFLPNVPRGDISCYPNTQGAIENGSTLPSERSNPVYAS
RTSMERNESSEVDVKPFVGLVNLNPEYASRTPVVQHESYQALLGSFHNNLFSPPNAP
RAAIACYPNTQGATGNGSTLPSDRVKPQYASRTSVEQNESSEVDVKPFVGLLNPNE
YASRTPVERNESSEIDVKPFVGLAIPNPEYAPRTPVERNESS

>TaAHL16

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EVPAGRDLVLSVAAFARRGRGALVLGAAGHVADVVLREPALVLRGTTEILSLAGC
FFPSPSASAAGVAVFLAGPRGSVLGGAVAEGGLVAAGPVVVMVATFVAAAIDRLPL
VKGDGSSKADGSDVHGVAGQWRCGGQPLQQQCGWAPLRRKLGAKS

>TaAHL17

MSFGKPDMSKEGMYQERKDAPATRFATPPPMHHRHAGHGDHQQQLECFSDDEVDS
RGTAEMKEPGGSAAGSQLVSGGGVGDGSSIEVAKKRRGRPPGSKNPKPPVVITRE
AEPAAAMRPHVIEIPGGRDVAEALARFASRRNLGICVLGTGAVGNVSLRHPCPTAP
GAAPAVVVFQGRFEILSISATFLPPAMAAVAPQAAAVAAGLSISLAGPHGQIVGGAV
VGPLYAATTIVVVAaftNPTFHRLPADDDAPMPVSVSLSGSAEADHRARQHQP
SQEQRHQRHVTPSAAQSVVEPCGVVPVYACHPQPQEVMWPLAARAPHQPPPPPPPY

>TaAHL18

MDPVA AAAAHGGGGHHFGPPVGTFFHPFHGHFPGQQRPLQQQHPAFQHFQDHHHQLLG
GMLAKQELVDESNTTINSGGSNNGNSGGEQHMGGGGGGGGGSAQQQQQHAG
GGGGEEGQQQQQQALAVMRRPRGRPAGSKNPKPPVIITRDSASALRAHVLEVAAG
CDVVDCAAFARRRQVGVCVLSGTGAVANVSVRQPGGAVATLAGCFDILSLSGSFL
PPPAPPSATGLTVYVSGGQGVVGGTVAGALVASGPVVIMAASFGNASYERLPLDD
EEPSAAQGLAQAHSSPPPLPLPTHQLQQQHQPSSSLAQAPDHHIPHNLMNGLQQLPG
DPYGWTTTPGGSGGRVSPY

>TaAHL19

MGSMGDHPLQGNHAYAHVPAGSNNDEDDASPPPSAGGGSSSGSRRRPRGRPPGSKN
KPKPPVVVTRESPNAMRSHVLEIASGADIVEAIAAFSRRRQRGVSVLSGSGAVTNVT
LRQPAGTGAAAVALRGRFEILSLSGAFLPAPAPPGATGLAVYLAGGQGVVGGSV
GELLASGPVMVIAATFGNATYERLPLDQDAEEGAVLSGSEGAATQLEQQGSGGA
AVPPPMYAVPQTTPSDVFGQWGQAAVARPPPTS

>TaAHL20

MANRWWDGHLGLPGVAPEQPSGSSGPKVESLALVVKDPETSPTS GGGEHADENKE
AVGGGEPRDLGAVVPAPNRRRPRGRPPGSKNPKPPIFVTRDSPNALRSHVMEVAGG
ADVCDIAIQFSRRRQRGVCVLSGAGTVANVALRQPSAPGGAVVALHGRFEILSLTG
TFLPGPAPPGSTGLTVYLAGGQGVVGGSVVGS LVAAGPVMVIASTFANATYERLP
LEDEDEGSGPPMAHDPLMAGGIHGHGHGHGMPDPSAAMPFMNIPPNNHGLGGGGD
GFPWAPHSRAPY

>TaAHL22

MGSMGDHPLQGNHGYAHVPAGSNNDEDDASPPPSAGGGSSSGSRRRPRGRPPGSKN
KPKPPVVVTRESPNAMRSHVLEIASGADIVEAIAAFSRRRQRGVSVLSGSGAVTNVT
LRQPAGTGAAAVALRGRFEILSLSGAFLPAPAPPGATGLAVYLAGGQGVVGGSV
GELLASGPVMVIAATFGNATYERLPLDQDAEEGAVLSGSEGAATQLEQQGSGGA
AVPPPMYAVPQTTPGDMFGQWGQAAVARPPPTS

>TaAHL23

MAGLDLGTNSYLHHHQLHLRHDDGAGGSDDGQDALSPGGGGGSAAGAGIGGS
EVVGRRRPRGRPPGSKNPKPPVIITRESANALRAHILEVAAGCDVFEALTAYARRRQ
RGVCVLSAAGTVTNVTLRQPQSAQSGPASPAVATLHGRFEILSLAGSFLPPPAPPGAT
SLSAFLAGGQGVVGGSVAGALVAAGPVVVVASSFSNVAYERLPLEDGDEVVPTTP
PPGSDQAGGGMPFGVEPSGSAGAGLPFFNLPMGMPPMPVDGHNGWPGAAGGIER
PPFS

>TaAHL24

MDPVTASIHGHHLPFPFNTDRDFHHHLQQQQLHLKSEDDHGGGSPFGGHGTKHGHED
DENSGNGHGSSELALISPSGGGLQDGGENGSRPRGRPAGSKNPKPPIIITRDSAN
TLRTHVMEVAGGCDISESITAFARRRQRGVCVLSGAGTVTNVTLRQPASQGAVVAL
HGRFEILSLSGSFLPPPAPPEATGLTVYLAGGKGQVVGTVVGSLSLAAGPVVIMAAS
FANAVYERLPLEEDELAAQGGADSTGMLAAGQQASQMAGGDVDPSEGLPPNL
LGNVQLPPEAAGCRWNPGTAGGRPSPF

>TaAHL25

MAGMDPGAGEPRGASHYLDLLLAQQQQSTPFSPFAHVKAEEHSMASPVRSASPAGA
GAGQHGGGADQQQPSSSAMVLADGGGGPARPMRRPRGRPPGSKNPKPPIIVMRDS
PNAFHSHILEVADGADIVECLAHEYARRRRRGVCVLSGAGVVTNVALRQPGAPSSSSP
GGLVATLQGGQFEILSLTGTVLPAPPAASSLAVYLAGWQGGVVGGSVVGQLVAAG
PVFLMAASFANAVYERLPLEGEAEEAGTATAATEAQGAAAAAQSPGALPQQPAAS
QSSEVTGGEAGGLGMPLCNLEGNVGSYHLPGP GDNLGSWSGVRP

>TaAHL27

MATGSSKWWQGPMDFPQPQPQQMQQHQLQHQLPLQPLQPAVTMPAPAPAVAAS
PESKQQQQQGGQGGEGQMGAAGAIVPLRRPRGRPMGSKNPKPPIIITRDS DALH
SHILEVAPGADVAACVAEYARRRRRGVCVLSGSGSVVDVVVRGAASAAPLPGRFE
LLSMTGTVLPAPPSEASGLAVMLSAGQGQVLGGCVVGPLVAAGPVTLFAATFANA
VYERLPLQDAADADV KPDLSAAPDASVPQEVQAQQPLAISQAMAMGAGYPDHRSP
QYPWGGHQGGGI

>TaAHL29

MGSM DGHPLQGNHAYAHVPAGSNNEDDASPPPSAGGGSSGSGRRRPRGRPPGSKN
KPKPPVVVTRESPNAMRSHVLEIASGADIVEAIAAFSRRRQRGVSVLSGSGAVTNVT
LRQPAGTGAAVALRGRFEILSLSGAFLPAPAPPATGLAVYLAGGQGQVVGGSVM
GELLASGPVMVIAATFGNATYERLPLDQDAEEGAVLSGSEGAATQLEQQGSGGAAV
PPPMYAVPQTPPSDMFGQWGQAAVARPPPTSF

Table S4-2. All primer sequence in this study

Gene name	Primer sequence (5'-3')	Purpose
qTaPAL	F: GCCATTGACATACTGAAG R: ATTCTTGACATTCTCCTCTA	qPCR
qTaBGLU11	F: GTGATGAAGAAGAATGTTG R: ATATACCGCAATGTAGTG	
qTaFAH	F: CGACATGACTGACATCTT R: GAGACCAAAGGTAAGTAA	
qTaHCT	F: ACAGATATGAGAGTGGTTAGT R: ATTCAGCACGCAACATAG	
qTaATCAD4	F: CTACACATACACCCTCAG R: GTTCTTAACCTGGTGGAC	
qTaCHS	F: GGACTACTACTTCAAGAT R: TATATCTCTTCTGATCTG	
qTaF3'H	F: CTACTACGCTAACCAACA R: CAACACCATCACATTCAA	
qTaFC6A	F: CTGGACGCTACATACTCC R: GAGATCAACCACAGGCAG	
qCYP98A3	F: GAATATACTCGTGGTACAATATGA R: ACTTGTGTCTCCCTGATT	
qTaAHL19	F: ATGGGGAGCATGGACG R: CTTGGGCTTGTTCTTG	

qTaF3GT	F: TTACGGAGCGAGGATTAC R: ATTACCCTTGTCCTCATAAC	
qF3GT-VIGS	F: GCAACTGCAATCCTCTGA R: AAAGGACACGTACACGAC	
β -Actin	F: GGAAAATCAGTCTCGGTTTCTAG R: TCATACAGCAGGCAAGCAC	
TaAHL19	F: ATGGGGAGCATGGACGGCCACCCGCT R: CTAGAATGACGTCGGCGGAGGCCG	Gene clone
ProF3GT	F: AAGCTGGAACCGTGGTCTG R: CAATAAGGAAGATTCACCTTACCA	
TaF3GT-LIC	F: ACACAAAGTCGGACCATTCTG R: TGTGAATAGCCTCTCGTTGAC	VIGS
TaAHL19-EcoRI- BamHI	F: ccggaattcATGGGGAGCATGGACGGCCACCCGCT R: cgcggatccCTAGAATGACGTCGGCGGAGGCCG	Y1H
ProF3GT- KpnI	F: cgagctcAAGCTGGAACCGTGGTCTG R: cggggtaccCAATAAGGAAGATTCACCTTACCA	

Chapter 5

Molecular mechanism of the salivary protein Sa6007 of grain aphid *Sitobion avenae* in the induction of wheat defense responses

Adapted from:

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Abstract

The grain aphid *Sitobion avenae* is one of the most economically important cereal aphids worldwide. Aphid salivary proteins are key regulators of plant-aphid interactions. Based on previous transcriptomic analyses, a candidate salivary elicitor Sa6007, was identified from *S. avenae*. In this study, delivery of Sa6007 into wheat leaves through a *Pseudomonas fluorescens*-mediated type III secretion system induced the accumulation of hydrogen peroxide and callose deposition, indicating activation of plant defense responses. Furthermore, overexpression of Sa6007 in wheat significantly enhanced both salicylic acid (SA) and jasmonic acid (JA)-mediated plant defense responses and consequently reduced aphid performance. Protein-protein interaction assays further demonstrated that Sa6007 directly interacts with the wheat transcriptional factor *TaTIFY10b* in the nucleus. Taken together, these findings demonstrate that Sa6007 acts as a salivary elicitor that is involved in activating the wheat defense response and interacting with *TaTIFY10b*, which provide novel insights into the aphid-wheat interactions and the molecular mechanism underlying the induced plant defense response.

Keywords: grain aphid, salivary elicitor, salicylic acid, jasmonic acid, defense response

1. Introduction

The previous chapters identified key defence components involved in both non-host and natural host resistances in wheat. However, successful defence activation depends not only on downstream defence responses but also on the initial perception of pest attack. Aphids are important phloem-feeding agricultural and horticultural crop pests in the world, causing serious yield losses on crops. Prior to feeding, aphid stylets penetrate the plant epidermis and mainly follow an intercellular pathway toward their feeding sites in sieve elements (Tjallingii and Esch, 1993). During probing and feeding, aphids secrete two types of saliva: gelling and watery saliva (Miles, 1999). The first hardens to form a sheath around the stylets with a protective function, and the latter contains complex proteins (e.g. salivary effectors) that can be injected into the plant vascular system (Kettles and Kaloshian, 2016). Saliva plays an important role in the interaction between aphids and host plants (Van Bel and Torsten, 2016). During a long period of coexistence, plants have developed strategies for countering herbivores. Recent evidence suggests that some conserved molecules in the aphid saliva are similar to pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) on the cell surface, which trigger PAMP-triggered immunity (PTI). Aphid then secretes effector proteins into the host plant to interfere with PTI, resulting in effector-triggered susceptibility (ETS). However, some plants can evolve a resistance gene (R gene) encoding the related resistance proteins that recognize effectors and trigger effector-triggered immunity (ETI), further preventing aphid from infestation.

A previous study found that treatment of *Arabidopsis thaliana* with 3-10 kDa watery saliva from *Myzus persicae* led to a significant decrease in aphid fecundity (Vos and Jander, 2009). Similarly, the infiltration of watery saliva from *Sitobion avenae* into wheat leaves significantly upregulated the expression of SA-related genes, such as *PAL* and *PR1*, thereby having an adverse effect on aphid feeding behavior and development (Zhang et al., 2017a). These results demonstrated that that salivary proteins of *S. avenae* involved in induction of wheat defense. Salivary proteins are generally classified into two functional categories: elicitors, which activate plant defense responses, and effectors, which suppress host immunity (Hogenhout and Bos, 2011; Snoeck et al., 2022). Type III secretion system (T3SS) of *Pseudomonas fluorescens* EtAnH was successfully employed to deliver potential salivary effectors into wheat leaves for transient overexpression (Zhang et al., 2023; 2024), offering support for functional analysis of aphid salivary proteins in wheat. Five salivary effectors and two salivary elicitors were also identified in *S. avenae* (Li et al., 2022; Zhang et al., 2023; 2024; Fu et al., 2023).

The grain aphid *S. avenae* is the dominant species in Huang-huai-hai wheat region in China. *S. avenae* can cause huge yield losses by directly sucking phloem sap and transmitting plant viral disease, such as *barley yellow dwarf virus* (BYDV). At present, the main control of wheat aphid is chemical pesticides. Breeding and utilization of aphid-resistant varieties is an important approach for sustainable management of wheat aphids. But there is little understanding of aphid-wheat interaction mechanism,

and there is a lack of aphid-resistant wheat resources in production. In previous study, 526 transcripts were identified and predicted encoding secretory proteins using transcriptomic analysis of *S. avenae* salivary glands, and 114 salivary proteins were identified in the watery saliva (Zhang et al., 2017b; 2021). Identifying wheat aphid salivary elicitors and investigating their functions and mechanisms in regulating wheat defense responses is of great significance. In this study, the potential salivary elicitor *Sa6007*, which is highly expressed in salivary glands, was investigated for its role and molecular mechanism in regulating wheat defense responses. The study will provide important theoretical and practical significance for revealing aphid-wheat interactions and cultivating aphid-resistant wheat varieties.

2. Materials and methods

2.1. Aphids and plants

Winter wheat (*T. aestivum*) cultivars Mingxian169 were planted in organic soil (peat: vermiculite = 3:1) and grow in a climate chamber at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under 16 h light/8 h dark photoperiod for 12 days. Tobacco plants *N. benthamiana* planted in laboratory conditions (16 h light/8 h dark cycle, $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and after 4-5 weeks, the *N. benthamiana* were used in subcellular localization experiments using *A. tumefaciens*. The wheat aphid clones *S. avenae* were originally collected from a wheat field in Langfang City, Hebei Province, China ($39^{\circ}31'\text{N}$, $116^{\circ}42'\text{E}$) and reared for >3 years on wheat under laboratory conditions (16 h light cycle, $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$).

2.2. Sequence analysis

The signal peptide of *Sa6007* was identified by using SignalP 5.0 (<https://services.healthtech.dtu.dk/service.php?SignalP-5.0>). Multiple sequence alignments of *Sa6007* were performed using ClustalOmega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). A phylogenetic tree was constructed by the neighbor-joining method via MEGA7.0 and boot-strap values were calculated as a percentage from over 1000 replications. The amino acid sequences of *Sa6007* and its homolog were listed in Table S5-1.

2.3. Subcellular localization in *N. benthamiana*

To perform subcellular localization assays, the *Sa6007* CDS excluding the signal peptide and *TaTIFY10b* CDS were cloned into the pCambia1300 vector using the In-Fusion Cloning Kit (Takara, Japan) according to the manufacturer's protocols and the primers were listed in Table S5-2. The subcellular localization assay was performed as described in Section 2.11 of Chapter 3.

2.4. Bacterial type III secretion system (T3SS)-mediated overexpression in wheat plants

The *Sa6007* sequence was cloned into the pEDV6 by gateway recombination (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocols using the primers listed in Table S5-2. The overexpression assay was performed as described in Section 2.15 of Chapter 3.

2.5. Detection of callose deposition and H_2O_2 accumulation in wheat leaves

After 4 days of infiltration, the leaves infiltrated with *P. fluorescens* EtHAN were fixated and destained in 1: 3 acetic acid/ethanol (v/v) solution overnight for the visualization of callose. The callose deposition in wheat leaves was detected with aniline blue according to previously reported protocols (Hood and Shew, 1996). Echo Revolve Hybrid Microscope (Echo Laboratories, San Diego, CA) was used for observation and photography. H_2O_2 accumulation was examined in infiltrated wheat leaves by 3'-diaminobenzidine (DAB) staining described by Zhao et al. (2018). The stained leaves were photographed using Olympus SZX-16 (Olympus Corporation, Tokyo, Japan). Three independent biological replicates were performed for each treatment.

2.6. Analyses of SA and JA contents

Jasmonic acid (JA) and salicylic acid (SA) levels were determined as described in Section 2.7 of Chapter 3.

2.7. RT-qPCR

Expression levels of *Sa6007* in different aphid stages were examined using RT-qPCR analysis. For the analysis of the *Sa6007* spatio-temporal expression profile, twenty apterous adults of *S. avenae* were fed on wheat leaves for 0 h, 6 h, 12 h, 24 h and 48 h, and then collected to analyze the expression levels of *Sa6007*. The expression levels of SA and JA-related defense genes, including *phenylalanine ammonia-lyase* (*PAL*), *pathogenesis related protein-1* (*PR1*), *lipoxygenase* (*LOX*) and *fatty acyl desaturase* (*FAD*) of wheat overexpressing pEDV6: *Sa6007* and pEDV6: *DsRed* were also detected. RT-qPCR were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). RNA extraction and qRT-PCR were performed as described in Section 2.9 of Chapter 3. Primers were designed using Beacon Designer 7 software and listed in Table S5-2. β -actin and *NADH hydrogenase* were used as internal reference genes (Zhang et al., 2017a; Yang et al., 2014). Three replicates were conducted for each treatment.

2.8. Aphid bioassays

After 2 days of infiltration, two alate adult *S. avenae* were transferred to infiltrated leaves within transparent plastic clip cages (2.5 cm×2.5 cm×2.5 cm). After 24 h, all adults and other nymphs were removed, leaving only one new-born nymphs on each treated leaf. The number of surviving aphids and the number of newly produced nymphs were recorded daily for 21 days. After each 4-day period, the wheat plants were replaced by freshly infiltrated wheat leaves. The pEDV6: *DsRed* was set as a control. A total of fifty-five independent biological replicates were performed for each treatment.

2.9. Yeast two-hybrid assays

Wheat cDNA libraries that infested by aphids at different feeding times were used as described previously (Zhang et al., 2022d). The CDS of *Sa6007* corresponding to the mature protein was cloned into the pGBKT7 vector to generate *Sa6007*-BD (bait vector), and wheat putative *Sa6007*-interacting proteins were screened using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, USA) according to the manufacturer's protocols. To further confirm the interaction between *Sa6007* and *TaTIFY10b*, the yeast two-hybrid (Y2H) gold yeast strain was co-transformed with *Sa6007*-BD and *TaTIFY10b*-AD constructs, and serial dilutions of the transformed cells were plated onto appropriate selective medium SD/-Leu/-Trp, SD/-His/-Leu/-Trp, SD/-Ade/-His/-Leu/-Trp, respectively. The primers used in this study are listed in Table S5-2. Yeast strains co-transformed with pGBKT7-53 and pGADT7-T, pGBKT7-Lam and pGADT7-T were set as positive and negative control mating, respectively.

2.10. Bimolecular fluorescence complementation (BiFC) assay

The plasmids used in BiFC assay were constructed using Gateway technology (Invitrogen) according to the manufacturer's protocol. Firstly, the CDS of *Sa6007* and *TaTIFY10b* were cloned into the pDONR221 entry vector using the BP Clonase reaction mix (Thermo Fisher Scientific, Waltham, MA, USA) to create the entry clones. The primers used are listed in Table S5-2. After verification by sequencing, the *Sa6007* and *TaTIFY10b* clones were mobilized into pGTQL1221-YN and pGTQL1221-YC respectively. The recombinant constructs were transformed into the agrobacterium strain GV3101. Agrobacterium cultures were resuspended in infiltration buffer [10mM MgCl₂, 10mM MES (pH 5.6), 0.1mM AS] at an OD600 of 0.6 before used. The constructs were transiently expressed in *N. benthamiana* leaves for 36h. Fluorescence signals for each combination were visualized with an inverted confocal microscope (Zeiss LSM 980). YFP was excited at 514 nm and captured at 565-585 nm.

2.11. Immunoprecipitation and immunoblotting analysis

Sa6007-HA and *TaTIFY10b*-MYC were co-expressed in *N. benthamiana* leaves, and total protein was extracted from these leaves using the RIPA Lysis Buffer (Beyotime, China). Protein extracts were centrifuged at 12000 g at 4 °C for 30 min and the supernatants were incubated with anti-HA magnetic beads or anti-Myc magnetic beads (Thermo Fisher, USA) overnight at 4 °C. The Co-IP assay was performed using the Pierce Magnetic HA-Tag IP/Co-IP Kit and Pierce Magnetic Myc-Tag IP/Co-IP Kit (Thermo Fisher, USA). The immunoprecipitated proteins were detected via Western blotting using anti-Myc (1: 1000) antibody or anti-HA antibody (1: 1000) (antibodies-online Inc., Germany). The luminescent signal was visualized using BIO-RAD ChemiDocTMXRS+. *N. benthamiana* leaves co-expressed with *TaTIFY10b*-Myc and HA were set as controls.

2.12. Statistical analysis

Multiple comparisons were performed using GraphPad Prism with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Pairwise comparisons were performed by two-tailed Student's *t* test. Differences were considered statistically significant at $P \leq 0.05$, with $P \leq 0.01$ indicating highly significance.

3. Results

3.1. Characterization of *Sa6007* candidate elicitor in *S. avenae*

The total length of the *Sa6007* gene contains a 642 bp open reading frame, encoding 214 amino acids, and the predicted molecular weight is 24.43 kDa. The first 20 amino acids constitute the signal peptide, with cleavage predicted between residues 20 and 21. Eleven proteins with high homology similarity to *Sa6007* were selected by BLAST analyses. The results showed that *Sa6007* has the highest identity with the *Acyrtosiphon pisum* (94.39%) ortholog but only 51.39% and 48.60% sequence identity to *Aphis gossypii* and *A. glycines* orthologs (Figure 5-1A). Phylogenetic analysis showed that *Sa6007* and the *Metopolophium dirhodum* ortholog were closely related, clustering into an independent clade (Figure 5-1B).

The expression levels of *Sa6007* in different instars of *S. avenae* were also different, with the highest expression levels in the first instar and second instar, and significantly higher than those in age-stage aphid ($P < 0.05$) (Figure 5-1C). Based on RT-qPCR, *Sa6007* was expressed at different feeding times of wingless adults, and the expression level reached the highest at 24 h and 48 h after feeding, which was significantly higher than other feeding times ($P < 0.05$) (Figure 5-1D).

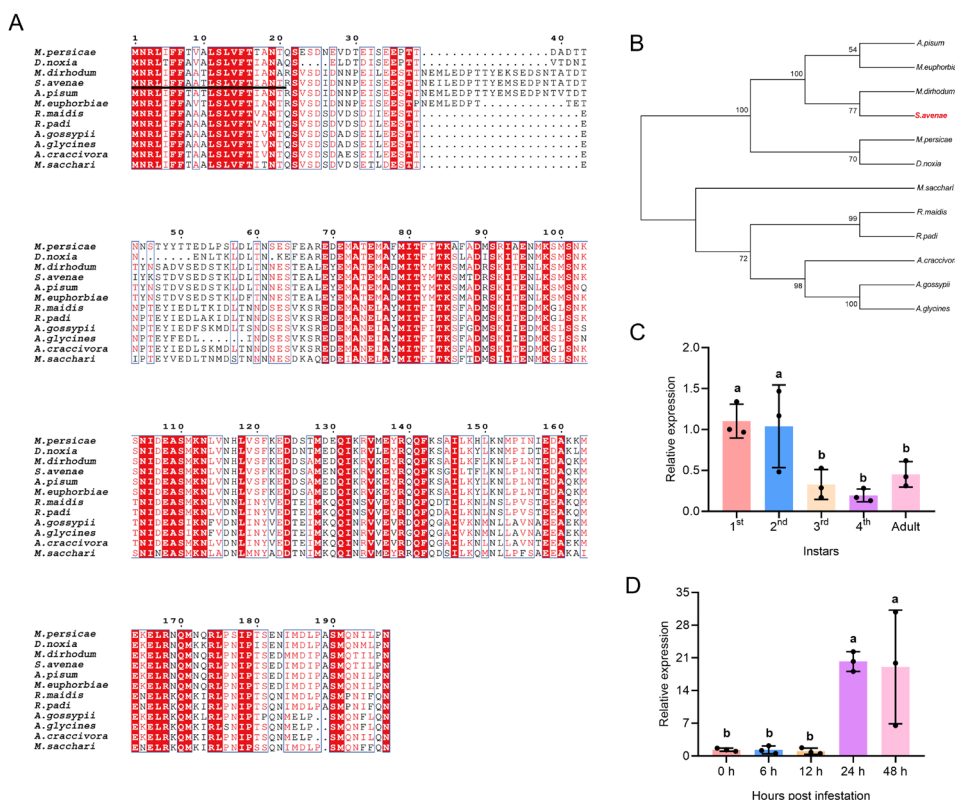


Figure 5-1: Characterization of Sa6007 in *Sitobion avenae*. (A) Multiple sequence alignment of the Sa6007 protein and orthologs from other aphid species. The deduced amino acid sequences from eleven aphid species, including *Acyrtosiphon pisum* (NP_001233038.1), *Metopolophium dirhodum* (XP_060862023.1), *Macrosiphum euphorbiae* (CAI6350678.1), *Myzus persicae* (XP_022172257.1), *Diuraphis noxia* (XP_015367067.1), *Rhopalosiphum maidis* (XP_026807022.1), *Aphis craccivora* (KAF0757856.1), *Rhopalosiphum padi* (XP_060842774.1), *Melanaphis sacchari* (XP_025202471.1), *Aphis gossypii* (XP_027836370.1) and *Aphis glycines* (KAE9527358.1). Red shades indicate identical amino acids. Alignment was created using Clustal Omega. (B) Phylogenetic tree constructed by comparing the amino acid sequences of Sa6007 and the orthologs from other aphid species. A phylogenetic tree was constructed by the neighbour-joining method using MEGA7.0. Bootstrap values calculated as a percentage for 1000 replications are shown at the nodes. (C) Relative expression levels of *Sa6007* in different instars of *S. avenae*. The relative expression of the 1st instar was used as the control. (D) Relative expression levels of *Sa6007* in apterous adults of *S. avenae* feeding on wheat leaves for different times. The expression level at 0 h was used as the control. Standard error (SE) is represented by the error bar. Different letters above bars indicate significant difference in the relative expression level ($P < 0.05$, Duncan's multiple range test).

3.2. Subcellular localization of Sa6007 in *Nicotiana benthamiana*

To identify the subcellular localization of Sa6007, transient expression analyses of the Sa6007-GFP fusion protein in *N. benthamiana* leaves were performed. The control expressing GFP exhibited fluorescence throughout the whole cell (cytoplasm and nucleus). But the fluorescence of the Sa6007-GFP fusion protein was only observed in the nucleus of *N. benthamiana* cells (Figure 5-2), which suggested that Sa6007 encodes a nucleus-targeting protein.

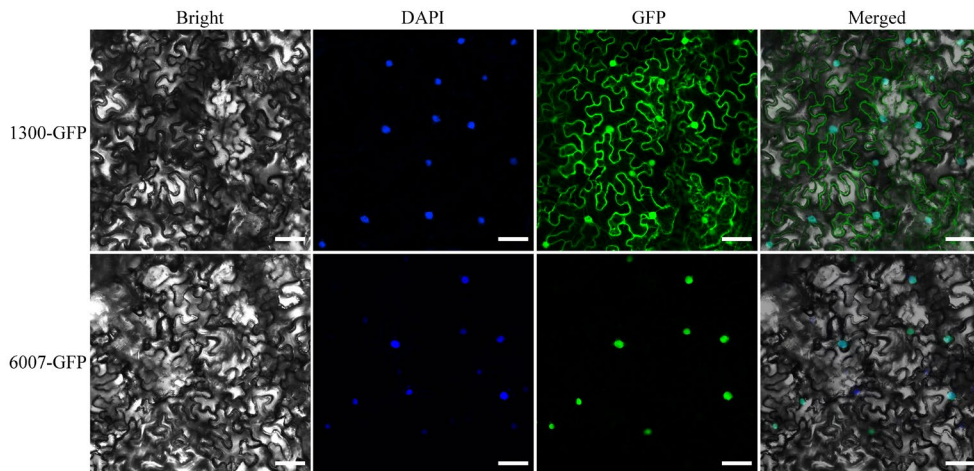


Figure 5-2: Subcellular localization of Sa6007 in plant. GFP (empty vector, pCAMBIA1300) and effector Sa6007 were transiently overexpressed in *N. benthamiana* leaves by *Agrobacterium* infiltration. The images were taken 3 days after agroinfiltration using confocal microscopy. Bar = 20 μ m.

3.3. Delivery of Sa6007 induced host plant defense response

To investigate the function of Sa6007 in inducing host plant defence, Sa6007 was cloned into the expression vector pEDV6 and then delivered into wheat leaves using the *P. fluorescens* EtHAN-mediated delivery system. Previous studies reported that infiltration of EtHAN expressing AvrRpt2, a well-characterized effector from *Pseudomonas syringae* was sufficient to induce in plant cell death and noticeable chlorotic phenotype in wheat leaves. As presented in Figure 5-3A and B, the AvrRpt2-inoculated wheat plants (positive control) displayed obvious chlorosis symptoms and hydrogen peroxide (H_2O_2) accumulation after 4 days, suggesting that the bacterial type III secretion system was successfully established in this study. The infiltration of EtHAN expressing Sa6007 also induced obvious chlorosis symptoms and H_2O_2 accumulation on leaves. In contrast, the infiltration of the EtHAN strain carrying pEDV6: *DsRed* into wheat did not result in any phenotypic changes or H_2O_2 accumulation on wheat leaves.

Aniline blue staining showed that callose deposition was present on wheat plants inoculated with EtHAN carrying pEDV6: *DsRed* and positive control pEDV6:AvrRpt2,

indicating the activation of PTI in wheat (Figure 5-3C). And more callose deposits were detected in wheat leaves expressing Sa6007 when compared to controls. These results suggested that Sa6007 stimulated PTI-related callose deposition in wheat plants.

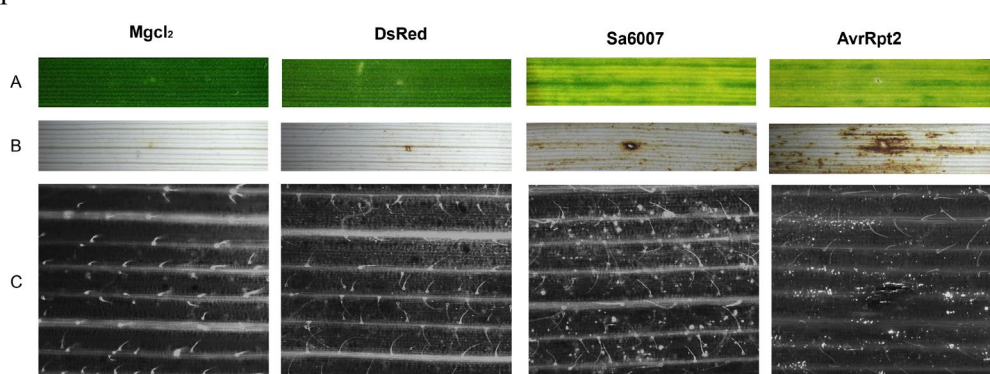


Figure 5-3: Effects of delivering Sa6007 into wheat on leaf phenotypes and plant response (A) Phenotypes of wheat leaves inoculated with MgCl_2 buffer (blank control), *Pseudomonas fluorescens* EtHAN carrying pEDV6:Sa6007, pEDV6: *DsRed* (negative control), and pEDV6:*AvrRpt2* (positive control) at 4 d after infiltration. (B) DAB staining for H_2O_2 accumulation in wheat leaves infiltrated with MgCl_2 buffer, *P. fluorescens* EtHAN carrying pEDV6: *DsRed*, pEDV6:Sa6007 and pEDV6:*AvrRpt2* at 4 d after infiltration. (C) Callose deposition observed by fluorescence microscopy in wheat leaves infiltrated with MgCl_2 buffer, *P. fluorescens* EtHAN carrying pEDV6: *DsRed*, pEDV6:Sa6007 and pEDV6:*AvrRpt2* at 4 d after infiltration after aniline blue staining.

3.4. Delivery of Sa6007 induced salicylic acid and jasmonic acid signaling pathways

Considering that the SA- and JA- pathways are major signalling pathways involved in most plant defences against insect herbivores, the key genes associated with SA and JA and the levels of plant defence hormones were studied upon delivery of Sa6007 in wheat for 4 d. The results showed that the transcript levels of *PAL* (2.92-fold) were significantly upregulated after treatment with Sa6007 at 4 d ($P < 0.05$) and the expression levels of *PR1* (4.04-fold) were significantly increased at 4 days compared with the control ($P < 0.01$; Figure 5-4A). Similarly, the expression levels of *LOX* (1.39-fold; $P < 0.05$) and *FAD* (2.65-fold; $P < 0.01$) were significantly upregulated at 4 d after Sa6007 infiltration (Figure 5-4A). Furthermore, LC-MS/MS results showed that delivery of Sa6007 in wheat significantly increased the levels of JA and SA at 4 d compared with the control, suggesting that Sa6007 is involved in the induction of both the SA and JA defence pathways in wheat plants (Figure 5-4B).

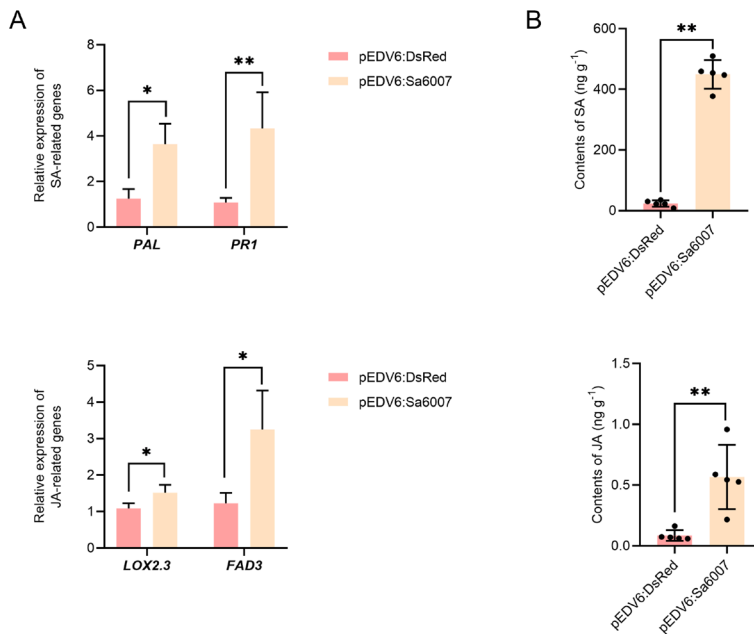


Figure 5-4: Delivery of Sa6007 in wheat induced SA-mediated and JA-mediated plant immunity. (A) The expression levels of the SA synthesis-related gene *PAL*, the SA signalling pathway marker gene *PR1* and JA synthesis-related genes *FAD3*, *LOX2.3* in wheat leaves after infiltration with *Pseudomonas fluorescens* EtHAN carrying pEDV6: *DsRed* or pEDV6:Sa6007 at 4 d. (B) Change in endogenous SA and JA contents in wheat leaves treated with *P. fluorescens* EtHAN carrying pEDV6: *DsRed* or pEDV6:Sa6007 at 4 d. Three biological replicates were performed for each treatment. Data are presented as the mean $\pm$ SE. Asterisks above the bars indicate significant differences between controls and treatments (* $P < 0.05$; ** $P < 0.01$; Student's *t* test).

3.5. Sa6007 expression in wheat enhances plant resistance against *S. avenae*

Wheat treated with *Sa6007* had a significant impact on the survival rate of aphids (Figure 5-5A). The survival rate of aphids on wheat leaves expressing Sa6007 was lower than that on control leaves. Also, the number of nymphs produced by aphid adults was also significantly lower on *Sa6007*-treated leaves than on the control leaves (Figure 5-5B).

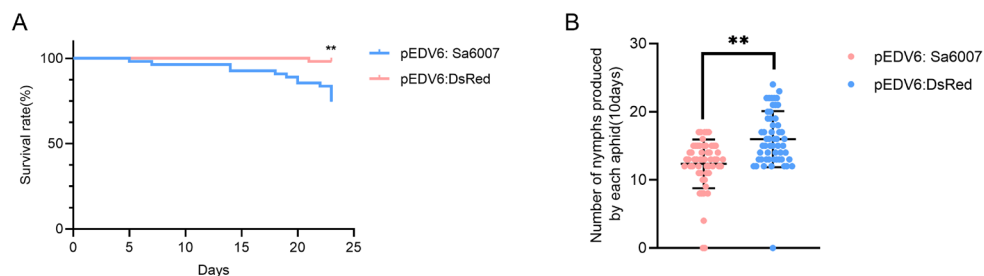


Figure 5-5: Survival and fecundity of *Sitobion avenae* after feeding on wheat leaves delivering with *Sa6007*. (A) Survival of *S. avenae* on wheat leaves expressing *Sa6007*. (B) Number of nymphs produced by *S. avenae* after feeding on wheat leaves infiltrated with pEDV6: *DsRed* and pEDV6:*Sa6007* for 10 days (n = 55). Asterisks above the bars indicate significant differences between controls and treatments (** $P < 0.01$, Student's *t* test).

3.6. *Sa6007* interacts with *TaTIFY10b* in plant cell nucleus

To identify the *Sa6007*-interacting proteins, Yeast two-hybrid (Y2H) assays were conducted using *Sa6007* as the bait to screen an aphid-infested wheat cDNA library. Firstly, we determined that the Y2HGold strain carrying pGBKT7: *Sa6007* could not grow normally on synthetic defined (SD) medium without tryptophan but supplemented with 5-bromo-4-chloro-3-indoxyl- α -D-galactopyranoside (X- α -Gal) and aureobasidin A (AbA) (single dropout, SDO/X/A), indicating that pGBKT7: *Sa6007* has no self-activation activity. In addition, the size of Y2HGold colonies expressing pGBKT7: *Sa6007* grown on SDO/X medium was consistent with that of colonies containing empty vector pGBKT7 (Figure 5-6A). Therefore, it could be used for further Y2H screening. A total of five CDS full sequences of putative positive targets of *Sa6007* were obtained preliminary (Table 5-1). As *Sa6007* localizes to the nucleus, we focused on the transcriptional factor of wheat *TaTIFY10b* (GenBank accession no. XM_044599969.1). Full-length *TaTIFY10b* CDS was used to generate the prey construct pGADT7: *TaTIFY10b* to confirm the interaction via Y2H assays. The yeast strains co-transformed with pGBKT7: *Sa6007* and pGADT7: *TaTIFY10b* grew normally on SD medium lacking tryptophan, leucine, histidine, and adenine with X- α -gal and AbA (quadruple dropout, QDO/X/A), suggesting that *Sa6007* interacted with *TaTIFY10b* in yeast cells (Figure 5-6B).

Table 5-1. *Sa6007* candidate interacting proteins

Number	Gene name	Gene accession number
1	Transcription factor <i>TaTIFY10b</i>	XM_044599969.1
2	CBS domain-containing protein CBSX3, <i>TaCBSX3</i>	XM_044508808.1
3	Glucose-6-phosphate 1-epimerase, <i>TaG6P1E</i>	XM_044532577.1
4	Universal stress protein PHOS34, <i>TaPHOS34</i>	XM_044548432.1
5	Ubiquitin-conjugating enzyme E22, <i>TaE22</i>	XM_044534910.1

Subcellular localization of *TaTIFY10b* was examined by transiently expressing the *TaTIFY10b*: GFP fusion in *N. benthamiana* leaves. The fluorescence signal of

TaTIFY10b-GFP was observed only in the nucleus of *N. benthamiana* cells (Figure 5-6C), indicating that TaTIFY10b localizes to the nucleus. The interaction between Sa6007 and TaTIFY10b were confirmed by the BiFC assay. The result showed that yellow fluorescence was detected in the nucleus of *N. benthamiana* leaves co-infiltrated with YN-Sa6007 and YC-TaTIFY10b, while no fluorescence was detected in the negative controls (Figure 5-6D), indicating that YN-Sa6007 and YC-TaTIFY10b are localized to and interact in the nucleus of plant cells. We further verified the interaction between Sa6007 and TaTIFY10b by *in vivo* co-immunoprecipitation (Co-IP) assays in *N. benthamiana* leaves. As shown in Figure 5-6E, in leaves co-infiltrated with Sa6007-HA and TaTIFY10b-Myc, the Sa6007-HA and TaTIFY10b-Myc proteins could be immunoprecipitated by anti-HA and anti-MYC agarose beads, respectively; however, no target protein was immunoprecipitated in the control groups. These results further confirmed the interaction between Sa6007 and TaTIFY10b *in vivo*.

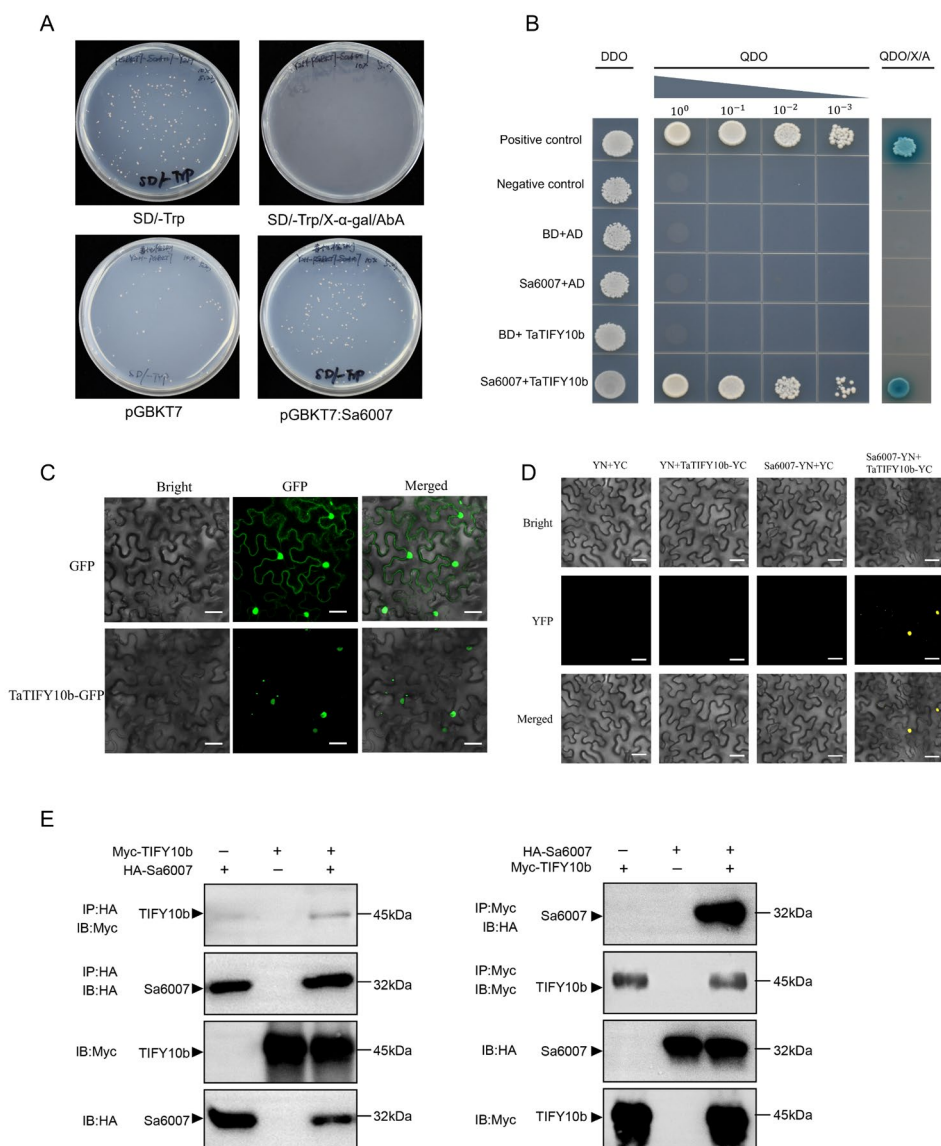


Figure 5-6: Salivary protein Sa6007 interactions with TaTIFY10b of wheat. (A) Self-activation and toxicity detection of pGBKT7:Sa6007. (B) Yeast two-hybrid assays to detect the interaction between Sa6007 (Bait) and TaTIFY10b (Prey). Gradient concentrations of the yeast cells co-transformed with Sa6007 and TaTIFY10b were assayed for growth on SD-Trp-Leu-His-Ade LB plates containing X-a-gal and aureobasidin A (QDO/X/A). Yeast cells co-transformed with pGBKT7-53+pGADT7-T and pGBKT7-Lam+pGADT7-T are used as positive and negative controls, respectively. (C) Subcellular localization of TaTIFY10b in *N. benthamiana*. The images were taken 2 days after agroinfiltration using confocal microscopy. Bar = 20 μ m. (D) BiFC assays for the interaction of Sa6007 with TaTIFY10b in *N. benthamiana*. YFP signals were introduced with a mixture of YN-Sa6007 and YC-TaTIFY10b construct. YFP signals

were observed at 36 h post-incubation using laser scanning confocal microscope. Infiltration with *Agrobacterium* expressing the Sa6007 or TaTIFY10b alone was used as controls. Scale bar = 20 μm . (E) Confirmation of the interaction between Sa6007 and TaTIFY10b using Co-IP assays. Western blots of total proteins from *N. benthamiana* leaves transiently expressing the labelled proteins eluted from anti-HA or anti-Myc magnetic beads were detected with the anti-Myc or anti-HA antibody. The sizes of Sa6007: HA and TaTIFY10b: MYC bands were 32 and 45 kDa, respectively.

4. Discussion

Salivary proteins secreted by aphids perform an important function in modulating plant defense (Elzinga and Jander, 2013; Yates and Michel, 2018). Several salivary proteins of *S. avenae* have been demonstrated to be involved in triggering wheat defense responses. For example, *S. avenae* salivary elicitor SmCSP4 enhance plant resistance to wheat aphids by interacting with TaWRKY76 to activate the SA signaling defense pathway in wheat (Zhang et al., 2023). Also, the small molecular protein GroES, derived from the primary endosymbiont *Buchnera aphidicola* of *S. avenae*, can induce ROS accumulation and callose deposition in wheat, thereby enhancing the resistance of wheat to aphids (Li et al., 2022). In this study, Sa6007, a salivary protein specifically expressed in the salivary glands of *S. avenae*, was found that the expression level was significantly up-regulated during aphid feeding on wheat plants, indicating that Sa6007 may be involved in the interactions between aphid and wheat. In addition, potential plant responses associated with the bacterial delivery system cannot be completely excluded.

S. avenae is a destructive herbivorous insect and feed on wheat plants. To investigate the roles of Sa6007 in regulating wheat defense, Sa6007 was delivered into wheat cells by EtHAN-mediated delivery system, which has been successfully applied to study the functions of salivary protein of wheat aphids (Zhang et al., 2022d, 2023). The delivery of Sa6007 in wheat leaves induced both H_2O_2 accumulation and callose deposition. A previous study showed that callose deposition was involved in the phloem defense mechanism that prevents the flow of nutrients to piercing-sucking insects (Will and van Bel, 2006). Salivary elicitor SmCSP4 also have been found to induce callose deposition (Zhang et al., 2023). Moreover, plant hormones JA and SA are important signaling conferring plant resistance against aphids (Züst and Agrawal, 2016). The expression levels of SA pathway-associated genes *PAL* and *PR1*; JA pathway-associated genes *FAD* and *LOX* were significantly up-regulated in Sa6007-infiltrated wheat leaves. In addition, both SA and JA content of wheat leaves was significantly increased after Sa6007 infiltration. Our results showed that delivery of Sa6007 into wheat induced both the JA and SA-dependent plant defense signaling pathways. Although the SA and JA signaling pathways have been proven to be in an antagonistic relationship (Thaler et al., 2012), some molecular modules of plants can activate or suppress the SA and JA signaling pathways simultaneously. For example, the transcriptional factor *WRKY70* inhibited plant response by suppressing the expression of SA and JA signaling pathway genes (Chakraborty et al., 2020). The overexpression of a cyclin-dependent kinase-like gene of *S. avenae* (SaCDK) in wheat

decreased the expression levels of both JA- and SA-related genes and enhanced its susceptibility against aphids (Zhang et al., 2024). It is speculated that Sa6007 interacts with the components of wheat plants that are involved in modulating both JA and SA defensive pathways.

Although T3SS-mediated delivery system enables the delivery of aphid salivary proteins into plant cells and provides a useful approach for evaluating their potential biological activities, it does not fully reproduce the natural delivery of salivary proteins during aphid feeding. In particular, the amount, spatial distribution, and temporal dynamics of Sa6007 delivered through the T3SS may differ from those occurring during aphid probing and feeding. Therefore, the T3SS results should primarily be interpreted as evidence that intracellular delivery of Sa6007 is sufficient to trigger defense-associated responses in wheat.

TIFY transcription factors, originally named ZIM proteins (Zinc-finger protein expressed in Inflorescence Meristem) (Nishii et al., 2000), are involved in regulating plant growth, development, defense, and stress response processes (Zhang et al., 2012; Aparicio et al., 2013). The transcription factor TIFY10b belongs to JAZ protein of the TIFY family, possessing a TIFY motif and a conservative Jas domain at the C-terminus. TIFY family genes have been found to play an important role in responding abiotic stresses. For example, overexpression of *GsTIFY10a* in *Arabidopsis* significantly increases the expression of stress-related genes and enhances the plants resistance to alkaline stress during seed germination, the seedling stage, and the adult stage (Zhu et al., 2011). Also, overexpression of *OsTIFY11a* in rice leads to increased tolerance to high salt and drought stress (Ye et al., 2009). In our study, full-length Sa6007 interacted with TaTIFY10b in the nucleus. A previous study showed that TaTIFY10b was implicated in regulation of grain weight and the signaling pathway of TaSnRK2.4 controlling grain size through phosphorylation of TaTIFY10b (Chen, 2021). Nevertheless, the knowledge that TIFY10b is involved in the process of biological stress have rarely been characterized to date.

TIFY, as a novel transcription factor in plants, there are more studies on the subfamily members JAZ proteins. JAZ protein, as a core negative regulator of the jasmonic acid signaling pathway, regulates the JA response through a COI1-mediated protein degradation mechanism and plays a key role in plant growth and defense (Zhang et al., 2012). A previous study demonstrated that *CsJAZ11* positively regulates tea plant resistance to *Colletotrichum camelliae*. Silencing *CsJAZ11* reduced disease resistance to *C. camelliae*, whereas overexpressing *CsJAZ11* increased it (Fan et al., 2025). Overexpression of *TaJAZ1* increases bread wheat resistance to powdery mildew through promoting reactive oxygen species accumulation (Jing et al., 2019). Currently, several TIFY proteins with unknown functions have been identified in plants, but research on TIFY proteins has mainly focused on plant stress responses (such as drought and salinity). In the future, more attention will be paid to their role in regulating herbivorous insects feeding.

The findings of this chapter may also provide a potential link between aphid salivary elicitor-triggered responses and the induced metabolic defenses identified in Chapter 4. Together, these findings raise the possibility that aphid-derived salivary signals

such as Sa6007 may contribute to the activation of downstream metabolic defenses during wheat–aphid interactions. However, whether Sa6007 is directly involved in regulating flavonoid biosynthesis remains unknown. To address this question, future studies will delivery Sa6007 in the aphid-resistant wheat cultivar HM6172 and study the related defense responses. In particular, flavonoid accumulation will be examined. Further investigation of the effects of Sa6007 on the *TaAHL19-TaF3GT*-rutin module may help establish a mechanistic connection between salivary elicitor perception and flavonoid-mediated aphid resistance in wheat.

To conclude, our findings provide the evidence that *S. avenae* employs the elicitor protein Sa6007 to enhance wheat resistance against aphid by inducing JA- and SA-related signaling pathways to manipulate plant defense responses. In addition, the functional mechanism of the wheat interaction protein TIFY10b, which interacts with Sa6007, in defending aphids is worthy of further study. These results provide new insights into the molecular mechanisms underlying cereal aphid–plant interactions.

5. Supplementary file

Table S5-1. All amino acid sequences of Sa6007 and its orthologs used for phylogenetic tree construction:

>*Sitobion avenae*

MNRLIFFAATLSLVFTIANTRSVSDIDNNPEISEESTTNEILEDPTTYEMSEDPNTATDT
IYKSTDVSEDSTKLDLTNNESTEALAYEMATEMADMITYMTKSMTDRSKITENLKS
MSNKSNIIDEASMKNLVNHLVSFKEDDSAMENQIKRVKEYRQQFKSGILTFLKNLPL
NTEDAQKMEKELRNQMNQRLPNIPTSEDIMDIPASMQTILPN

>*Acyrtosiphon pisum*

MNRLIFFTATLSLVFTIANTRSVSDIDNNPEISEESTTNEMLEDPTTYEMSEDPNTVTD
TTYNSTDVSEDSTKLDLTNNESTEALAYEMATEMADMITYMTKSMADRSKITENLK
SMSNQSNIIDEASMKNLVNHLVSFKEDDSAMEDQIKRVKEYRQQFKSAILKYLNLP
LNTEDAQKMEKELRNQMNQRLPNIPTSEDIMDIPASMQNILPN

>*Metopolophium dirhodum*

MNRLIFFAATLSLVFTIANARSVSDIDNNPEILEESTTNEMLEDPTTYEKSEDSNTATD
TTYNSADVSEDSTKLDLTNNESTEALAYEMATEMADMITYMTKSMADRSKITENLK
SMSNKSNIIDEASMKNLVNHLVSFKEDDSAMEDQIKRVKEYRQQFKSAILKFLKNLP
LNTEDAQKMEKELRNQMNQRLPNIPTSEDMMDIPASMQTILPN

>*Macrosiphum euphorbiae*

MNRLIFFAVTSLVFTIANTRSVSDIDNNPEISEESTPNEMLEDPTTETTYNSTDVSED
STKLDFTNNESTEALAYEMATEMADMITYMTKSMADRSKITENLKSMSNKSNIIDEA
SMKNLVNHLVSFKEDDSAMEDQIKRVKEYRQQFKSAILKYLNLPNTEDAQKME
KELRNQMNQRLPNIPTSEDIMDIPASMQNILPN

>*Myzus persicae*

MNRLIFFTVALS LVFTTANTQSESDNEVDTEISEEPTTDADTTNNSTYYTTEDLPSLD
LTNSESFEAREDEMATEMAFMITFITKAFADMSRIAENMKSMSNKSNIIDEASMKNLV
NHLVSFKEDDSTMDEQIKRVMEYRQQFKSAILKHLKNMPINIEDAKKMEKELRNQM
NQRLPSIPTSENIMDLPAASMQNILPN

>*Diuraphis noxia*

MNRLTFFAVALSLVFTIANAQSELDTDISEEPTTVTDNINENLTKLDLTNKEFEARED
EMATEMAYMITFITKSLADISKITENMKSMSNKSNIIDEASMKNLVNHLVSFKEDDNT

MEDQIKRVLEYRQQFKSAILKYLKNMPIDTEDAKLMEKELRNQMKKRLPNIPISENI
MDLPASMQNMLPN

>*Rhopalosiphum maidis*

MNRLIFFAAALSLVFTVANTQSVSDSDVSDIIEESTTENPTEYIEDLTKIDLTNNDSE
SVKSREDEMANELAYMITFITKSFADMSKITEDMKGLSNKTNIDEASMKNLVNNLIN
YVEDTEIMKQQINSVVEYRDQFQDTILKNLNSLPVSTEEAKQMENELRKQMKIRLPN
IPTSQNIMDLPASMPNIFQN

>*Aphis craccivora*

MNRLIFFTAALSLVFTIVNTQSVSDSDAESEILEESTTENPTEYIEDLSKMDLTNNDSE
SVKSREDEMANEIA YMITFITKSFADMSKITEDMKGLSNKTNIDEASMKNLVNIN
YVEDTEIMKQQINRVVEYRDQFQGAILKNLNLAVNTEEA EKMEKELRKQMKIRLP
NIPTSQNMELPSMQNILQN

>*Rhopalosiphum padi*

MNRLIFFAAALSLVFTVANTQSVSDSDVSDILEESTTENPTEYIEDLAKIDLTNNDSE
SVKSREDEMANELAYMITFITKSFADMSKITEDMKGLSSKTNIDEASMKNLVNIN
YVEDTEIMKQQINSVVEYRDQFQDAILKNLNSLPVSTEEAKQMENELRKQMKIRLPN
IPTSQNIMDLPASMPNIFQN

>*Melanaphis sacchari*

MNRLIFFTAALSLVFTITNTQSVSDSDVDSETLDESTTEIPTEYVEDLTNMDSTNNNN
ESDKAQEIDEIANELAYMITFITKSFTDMSIITENMKSLSNKSNINEASMKNLADNLMN
YADDTNIMKQQINRVMEYRRQFQDSILKQMNLLPFSAEAAKAIENELRKQMKIRLPN
IPSSQNIMDLPMSQNFQFN

>*Aphis gossypii*

MNRLIFFAAALSLVFTIVNTQSVSDSDADSEILEESTTENPTEYIEDFSKMDLTSNDSE
SVKSREDEMANEIA YMITFITKSFGDMSKIHEDMKSLSSSTNIDEASIKNFVDNLIN YV
EDTEIMKQQINRVVEYRDQFQGAIVKNMNLLAVNAEEAEKMEKELRKQMKLRLPN
IPTPQNMELPSMQNFLQN

>*Aphis glycines*

MNRLIFFAAALSLVFTIVNTQSVSDSDADSEILEESTTENPTEYFEDLINDSESVKSRE
DEMANEIA YMITFITKSFADMSKIHEDMKSLSSSTNIDEASIKNFVDNLIN YVEDTEIM
KQQINRVVEYRGQFQGAIVKNMNLLAVNAEEAEKMEKELRKQMKIRLSNIPTSQN
MELPSMQNFLQN

Table S5-2. All primer sequence in this study

Gene name	Primer sequence (5'-3')	Purpose
qSa6007	F: TGCCACCGATACCATCTATA R: TTCATATTCCAGTGCTTCAGTT	qPCR
qTaPAL	F: CCACCCTGGACAGATTGAA R: ATGAGCGGGTTGTCGTTG	
qTaPR1	F: AGTGCAAGTCCACCCTCATC R: CTTGGCCTTGGTGATCTCAT	
qTaLOX	F: GACCAGCGAAACAACAACC R: GCATACAATAGCGGGAACAC	
qTaFAD	F: TCCCATTCCACCTACTGC R: GGA CTCACCAATCCGAGA	
qdsSa6007	F: GAAGATCCTACCACCTAC R: TCAGTTGATTCA TTGTTAGT	

qNADPH	F: TTAACGGTATAGATGATCAGCA R: CGAGGAGAACATGCTCTTAGAC	
β -Actin	F: GGAAAATCAGTCTCGGTTTCAG R: TCATACAGCAGGCAAGCAC	
Sa6007	F: ATGCGAAGCGTGTTCAGACATCG R: TTAATTTGGTAGTATAGTTTGCATACTTGC	Gene clone
TaTIFY10b	F: ATGGCGGCTTCCGC R: TTATTGGTTTGGCTTCAGGG	
T7-Sa6007	F: TAATACGACTCACTATAGGGTCGGAAGATCCTAATACTGCC R: TAATACGACTCACTATAGGGTAGTTCTTTTCCATTTTTTGTG	dsRNA
Sa6007-SacI-XbaI	F: cgagctcATGCGAAGCGTGTTCAGACATCG R: gctctagaTGGTAGTATAGTTTGCATACTTGCA	Subcellu lar localizat ion
TaTIFY10b-EcoRI-BamHI	F: CGGAATTCATGGCGGCTTCCGC R: CGGGATCCTTATTGGTTTGGCTTCAGGG	
Sa6007-NdeI-BamHI	F: cccatagATGCGAAGCGTGTTCAGACATCG R: gcgggatccTTAATTTGGTAGTATAGTTTGCATACTTGC	Y2H
QB-Sa6007	F: gcaggctcaggggatacATGCGAAGCGTGTTCAGACATCG R: tgggtgcagggcgatacATTTGGTAGTATAGTTTGCATACTTGCA	BiFC
QB-TaTIFY10b	F: gcaggctcaggggatacATGGCGGCTTCCGCGAGGCAGGGGG R: tgggtgcagggcgatacATGGCGGCTTCCGCG	
attB-Sa6007	F: ggggacaagttgtacaaaaagcaggcttcATGCGAAGCGTGTTCAGACATCG R: ggggaccactttgtacaagaaagctgggtcATTTGGTAGTATAGTTTGCATACTTGC	Deliveri ng by T3SS

Chapter 6

General conclusions, discussion and perspectives

1. General conclusions

Plant resistance represents one of the most effective and environmentally sustainable strategies for aphid management. However, the molecular mechanisms governing wheat–aphid interactions remain incompletely understood because resistance is regulated by multiple biological and molecular processes, ranging from signal molecule recognition recognition and hormone signaling to secondary metabolism and insect adaptation. A comprehensive understanding of these interconnected defense layers is essential for developing sustainable aphid-resistant wheat cultivars. In this thesis, wheat resistance to aphids was systematically investigated from three complementary perspectives, including non-host resistance, host plant resistance, and aphid salivary protein-triggered immunity. By integrating transcriptomics, metabolomics, molecular genetics, protein interaction analyses and insect bioassays, this study provides new mechanistic insights into how wheat recognizes aphid infestation, activates defense signaling pathways, regulates defensive metabolites and proteins, and ultimately suppresses aphid colonization.

The first major finding of this thesis is the elucidation of molecular mechanisms underlying wheat non-/poor-host resistance against aphids. Although non-host resistance has long been considered the most durable form of plant resistance, its molecular basis in cereal crops remains poorly characterized. By comparing host, poor-host and non-host interactions among different aphid species, this study demonstrates that poor-host interactions induce substantially stronger defense responses than compatible interactions. Comparative transcriptomic and phytohormone analyses consistently confirmed that jasmonic acid signaling is preferentially activated after *M. persicae* feeding. Furthermore, the Bowman-Birk trypsin inhibitor TaBBTI-1 was identified as an important downstream defense protein that significantly reduced aphid reproduction when expressed in wheat or supplemented in artificial diets. The observation that ectopic expression of *TaBBTI-1* also enhanced resistance against the chewing insect *H. armigera* further suggests that this protein contributes to broad-spectrum insect resistance. These findings provide direct evidence that activation of JA-dependent defenses represents an important feature distinguishing poor-host resistance from compatible wheat-aphid interactions.

The second major finding of this thesis is the identification of a transcriptional regulatory module underlying aphid resistance in hexaploid wheat. From the perspective of host resistance, aphid-resistant variety HM6172 and aphid-susceptible variety ND399 were identified through field and indoor investigation. Comparative transcriptomic and metabolomic analyses revealed that cv. HM6172 preferentially activates phenylpropanoid biosynthesis, flavonoid biosynthesis and jasmonate signaling following aphid infestation. Particularly, rutin was identified as a major defensive metabolite which accumulation significantly reduced aphid performance. Mechanistically, the transcription factor *TaAHL19* was demonstrated to directly activate the expression of the flavonoid biosynthetic gene *TaF3GT*, thereby promoting rutin accumulation and enhancing aphid resistance. These findings establish the *TaAHL19-TaF3GT*-rutin regulatory module as a previously uncharacterized

molecular mechanism controlling wheat resistance against aphids and provide valuable candidate genes for resistance breeding.

During aphid probing and feeding, proteins present in salivary glands can be recognized by plant pattern-recognition receptors, resulting in regulation of plant defense responses (Snoeck et al., 2022; Jones and Dangl, 2006). Whether these diverse salivary proteins from *M. persicae* and *S. avenae* are key factors specifically modulating JA-mediated induction in non-host resistance remains unclear. A deeper understanding of the function of salivary elicitors and effectors will be essential for elucidating the molecular mechanisms underlying aphid adaptation. Consequently, we identified Sa6007, a salivary elicitor that is highly expressed in the salivary glands of *S. avenae* and is capable of triggering defense responses in wheat. Sa6007 induced the accumulation of hydrogen peroxide and callose, and enhanced JA levels, thereby inhibiting aphid performance. Further protein-protein interaction assays demonstrated that Sa6007 interacts with the wheat transcription factor TaFITY10b. In addition, TaFITY10b was identified as a JAZ protein associated with JA. However, it is unclear whether TaFITY10b is involved in the Sa6007-mediated increase in JA levels. Future studies will investigate whether TaFITY10b is involved in the degradation of JA, which could help to elucidate the molecular mechanism underlying the interaction between Sa6007 and TaFITY10b.

In conclusion, the wheat cv. HM6172, which exhibits stable resistance to aphids, has strong potential for large-scale cultivation and breeding. TaBBTI-1 and rutin can serve as bioactive anti-insect compounds for wheat aphid management in the field, while Sa6007 may be developed as an elicitor to induce plant resistance. In addition, the transcription factor *TaAHL19* represents a promising candidate resistance gene for the development of aphid-resistant wheat cultivars. Collectively, this study provides both theoretical insights and practical approaches for the development of green and sustainable management against wheat aphids.

2. General discussion and perspectives

2.1. Jasmonic acid integrates immune signaling and defensive metabolism during wheat–aphid interactions

JA is widely recognized as a central regulator of plant defense against herbivorous insects. Since the discovery of the octadecanoid pathway, extensive studies have demonstrated that JA signaling orchestrates diverse anti-herbivore defenses, including the production of defensive proteins, secondary metabolites and herbivore-induced plant volatiles (HIPVs), thereby reducing insect performance and improving plant fitness (Howe and Jander, 2008; Erb and Reymond, 2019). Among these phytohormones, SA is generally associated with defense against pathogens and phloem-feeding insects, whereas JA has traditionally been regarded as the primary regulator of resistance against chewing herbivores. For example, the wheat aphid salivary protein SmCSP4 enhances plant resistance by activating salicylic acid defense (Zhang et al., 2023). Although JA and SA were previously considered to act antagonistically, previous studies have shown that wheat aphid infestation also

induces the expression of JA-related genes and elevates JA levels (Liu et al., 2020; Reddy et al., 2013). And research has shown that the JA pathway is considered particularly effective against aphids compared with the SA defense pathway (Züst and Agrawal, 2016). Exogenous JA application suppresses aphid populations on tomato and Brassica species (Jiao et al., 2022; Rasool et al., 2017). These findings indicate that JA plays a broader role in aphid resistance. Despite these advances, the precise function of JA in wheat resistance against aphids has remained elusive. One important reason is that previous studies have generally focused on individual systems, such as resistant cultivars, hormone treatments or single candidate genes, making it difficult to distinguish whether JA represents a core regulator of wheat immunity or simply one of several downstream responses.

The results presented in this thesis consistently support the involvement of JA-associated responses in wheat defense against aphids. This study found that JA is associated with diverse stages of wheat defense, including poor-host resistance, natural host resistance and elicitor-triggered immunity. One of the defining characteristics of JA signaling is its ability to regulate extensive transcriptional reprogramming through the COI1-JAZ signaling module, thereby activating numerous transcription factors and defense-related genes (Browse, 2009). In addition to directly influencing aphid behavior as a defensive substance, JA can also coordinate multiple downstream defensive performance.

A comprehensive understanding of the mechanisms by which JA mediates plant resistance to aphids is crucial for elucidating the molecular basis of wheat-aphid interactions. Proteinase inhibitors constitute an important class of defense proteins in plants and are frequently induced by JA signaling following herbivore attack (Farmer and Ryan, 1990). Here, the JA-mediated signaling pathways positively regulated TaBBTI-1 accumulation in wheat during poor-host interactions with *M. persicae*; however, the underlying mechanisms remain to be elucidated. In addition to activating defense proteins, JA signaling is also closely associated with the regulation of secondary metabolism. For example, JA signaling regulates the production of benzoxazinoids that deter aphid feeding and impair insect development in maize (Ahmad et al., 2011). In this study, the *TaAHL19-TaF3GT*-rutin module provides a mechanistic explanation for the enhanced resistance to aphids observed in wheat line HM6172. Notably, aphid feeding markedly elevates JA levels; however, whether JA correlates with the *TaAHL19-TaF3GT*-rutin module remains to be determined. In addition, transcriptome analysis of wheat seedlings and mature plants revealed that despite significant differences in DEGs, the JA defense pathway was still activated, indicating a persistent configuration of jasmonic acid signaling. This defense is particularly important for wheat, which is susceptible to aphid infestation at multiple growth stages.

The simultaneous regulation of multiple downstream defense pathways by JA makes the analysis of JA-regulated defense networks a promising prospect for wheat breeding. However, the upstream mechanisms controlling JA activation following aphid infestation are still poorly understood in wheat. In particular, the receptors responsible for recognizing aphid-derived elicitors and transmitting these signals to

the JA signaling pathway have not yet been identified. In addition, the salivary protein elicitor Sa6007 directly confirmed its interaction with the JAZ protein, indicating that JA is involved in the salivary protein-induced wheat defense response. But the molecular mechanism involved still needs further in-depth research.

2.2. Wheat resistance to aphids is established through dynamic defense mechanisms

Plant-aphid interactions are highly dynamic biological processes involving continuous interactions between the host plant and insect. Unlike chewing herbivores, aphids establish long-term feeding sites within the phloem and continuously manipulate host plants through the secretion of salivary proteins. Consequently, successful plant defense cannot rely on a single resistance mechanism but instead requires coordinated responses operating throughout different stages of aphid colonization.

The earliest stage of plant-aphid interaction is the recognition of aphid infestation. During probing, aphids inject saliva containing numerous proteins into host tissues before reaching the phloem. These salivary components not only facilitate feeding but also serve as molecular signals perceived by host plants. Identifying wheat aphid salivary elicitors and investigating their functions and mechanisms in regulating wheat defense responses is of great significance for identifying aphid-resistance genes. A previous study showed that infiltration of watery saliva from *S. avenae* into wheat leaves significantly upregulated the expression of SA-related genes, such as *PAL* and *PR1*, thereby having an adverse effect on aphid feeding behavior and development. These results demonstrated that salivary proteins of *S. avenae* can directly participate in the induction of wheat defense responses (Zhang et al., 2017a). Currently, two salivary protein elicitors of *S. avenae* have been identified. GroES, a salivary protein derived from aphid primary symbiont *Buchnera*, activated the SA and JA defense pathways in wheat (Li et al., 2022). Furthermore, the salivary protein SmCSP4 from *S. avenae* interacted with the transcription factor *TaWRKY76* to activate salicylic acid (SA)-mediated defense responses, thereby enhancing wheat resistance to aphids (Zhang et al., 2023). In this study, the salivary protein Sa6007 was identified as an elicitor capable of activating hydrogen peroxide accumulation, callose deposition and defense hormone signaling in wheat. Furthermore, Sa6007 was shown to interact with the wheat transcription factor *TaTIFY10b*, suggesting that wheat rapidly perceives aphid-derived molecules and initiates intracellular immune signaling after infestation. Future studies should focus on elucidating the biological function of *TaTIFY10b* and uncovering the molecular mechanism by which the Sa6007-*TaTIFY10b* interaction enhances wheat resistance to aphid infestation.

Following initial recognition, wheat activates inducible defense responses that influence aphid feeding behaviour and host acceptance. Comparative analyses of compatible and poor-host interactions revealed that *M. persicae* induced stronger defense responses than the wheat aphid *S. avenae*. These observations indicate that the outcome of aphid colonization is determined not only by aphid adaptation but also by the ability of the host to rapid inducible defenses after infestation. These findings

revealed that poor-host resistance functions during the early stages of colonization by reducing aphid feeding efficiency and preventing successful host adaptation.

If aphids successfully overcome these initial barriers and establish phloem feeding, wheat subsequently deploys a third level of defense that limits insect growth and population development. In the resistant wheat cv. HM6172, transcriptomic analyses demonstrated that aphid infestation induced large-scale activation of genes associated with phenylpropanoid metabolism, flavonoid biosynthesis and other defense-related pathways. Targeted metabolomic analyses further revealed significant accumulation of rutin following aphid attack, while functional analyses confirmed that both rutin and the transcriptional regulator *TaAHL19* contribute substantially to aphid resistance.

Interestingly, transcriptomic analyses conducted at different developmental stages further support the concept of dynamic defense regulation. In this study, transcriptome sequencing was employed not only to investigate wheat responses to infestation by different aphid species under controlled environmental conditions, but also to explore aphid-induced defense responses under field conditions. These analyses encompassed two critical developmental stages of wheat, namely the seedling stage and the mature stage. The DEGs identified at the two developmental stages of wheat provide distinct insights into wheat defense strategies against aphid infestation. During the seedling stage, aphid attack predominantly induced phytohormone-mediated defense pathways, particularly the JA signaling pathway. This stronger activation of JA-related defenses may reflect a more robust perception and transduction of defense signals in young wheat seedlings following aphid infestation. In contrast, during the mature stage, aphid infestation not only altered phytohormone levels but also triggered substantial changes in flavonoid metabolism. As wheat experiences prolonged growth alongside continuous aphid infestation, defensive secondary metabolites are likely to play an increasingly important role at this stage by contributing to sustained chemical defense against herbivory. Overall, these findings suggest that wheat resistance to aphids is orchestrated through a dynamic and multilayered defense network, in which the relative contributions of phytohormone signaling and secondary metabolism vary across developmental stages.

Aphid colonization is a multistep process involving host location, probing, stylet penetration, phloem feeding and population establishment. Accordingly, resistance operating at only one stage may be insufficient to provide effective protection if aphids successfully overcome that particular barrier. In contrast, sequential defense mechanisms acting throughout the colonization process create multiple physiological obstacles that collectively reduce aphid fitness. Early recognition limits successful establishment, inducible defenses interfere with feeding behaviour, and prolonged metabolic responses suppress reproduction and population expansion. The integration of these temporally coordinated responses therefore provides a more robust defense system than any individual mechanism operating in isolation.

Recently, RNA interference (RNAi) has become a practical strategy for silencing target genes and holds great potential as a pest control strategy (Niu et al., 2021; Yan et al., 2020). Utilizing dsRNA to specifically silence key genes essential for the growth and development of pests not only facilitates pest management but also poses

low risk to non-target organisms, especially natural enemies of pests. A previous study has shown that silencing *SaZFP* of *S. avenae* in wheat using RNAi significantly reduced the aphid survival and reproductive rates, confirming that *SaZFP* is involved in the feeding and digestion processes of *S. avenae* (Sun et al., 2019). In addition, nanocarrier-mediated RNAi has successfully identified several genes that play a crucial role in the growth and development of wheat aphids, which can serve as potential RNAi targets for the control of wheat aphids. For example, silencing the salivary effector genes *Sg2204* in *S. graminum* and *Sm9723* in *S. avenae* effectively controls aphid population growth (Zhang et al., 2022d; 2022e). Furthermore, using dsRNA to successfully knock down the amino acid metabolic relay genes *ilvA* and *ilvE* of both the symbiotic bacteria and *S. avenae* leads to a significant decrease in aphid survival rate and fecundity (Li et al., 2023a, b). Clustered Regularly Interspaced Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) is a powerful tool for efficient site-directed genome editing. CRISPR/Cas9-mediated gene editing has been successfully applied to insects, such as pea aphid (Shigenobu et al., 2025). Studies have shown that knocking out *Laccase2* gene in *Acyrtosiphon pisum* using CRISPR/Cas9 revealed that *Laccase2* is crucial for the survival of aphids during their overwintering diapause (Shigenobu et al., 2025). However, this method has not yet been successfully applied to wheat aphids. In the future, the application of CRISPR/Cas9 in wheat aphids will provide a powerful tool for studying the functions of key genes involved in the interaction between aphids and wheat.

In addition, the findings presented in Chapters 4 and 5 suggest a potential regulatory framework underlying wheat resistance to *S. avenae*. Although these two regulatory modules were investigated independently, they may represent sequential or interconnected events during the wheat-aphid interaction. A plausible model is that aphid-derived salivary effectors, including Sa6007, are perceived by wheat cells and initiate early defense signaling through transcriptional regulators such as *TaTIFY10b*. This signaling may subsequently activate downstream transcription factors, including *TaAHL19*, thereby promoting *TaF3GT* expression, rutin biosynthesis, and enhanced resistance to aphid infestation. However, this hypothetical regulatory cascade has not yet been experimentally validated. In particular, whether Sa6007 can induce *TaAHL19* expression in the resistant cultivar variety HM6172 and whether *TaTIFY10b* functions upstream of *TaAHL19* remain important questions for future investigation. Based on the findings of this thesis, we propose a preliminary model in which aphid salivary effectors serve as upstream signals that activate host transcriptional regulatory networks, ultimately promoting flavonoid biosynthesis and aphid resistance. Future genetic and biochemical studies will be required to validate the molecular connections among Sa6007, *TaTIFY10b*, *TaAHL19*, and *TaF3GT*.

2.3. From molecular mechanisms to sustainable aphid-resistant breeding

Developing wheat cultivars with durable resistance to aphids remains one of the most sustainable approaches for reducing insecticide dependence and maintaining stable wheat production. However, despite decades of breeding efforts, progress in

developing aphid-resistant wheat cultivars has remained relatively slow. A major challenge is that aphid resistance is typically a quantitative trait governed by multiple genes and is further influenced by the complex interactions among the host plant, the insect pest, and the environment (Smith, 2005; Smith and Chuang, 2014). Moreover, aphids possess remarkable adaptive potential, allowing them to rapidly overcome resistance deployed in commercial cultivars. Consequently, these findings indicate that breeding strategies based exclusively on single resistance genes are unlikely to achieve durable and broad-spectrum aphid resistance.

Among emerging strategies, non-host resistance has attracted increasing attention because it represents one of the most durable forms of natural plant immunity. The widespread adoption of genome editing and omics methods provides a strong guarantee for the more precise and effective use of NHR-related traits in agriculture. Utilizing non-host resistance genes offers a novel approach to breeding broad-spectrum and durable resistance varieties. Non-host-implicated genes have been successfully applied in plant breeding against pathogens (Zhao et al., 2005; Sendín et al., 2012). For example, the *Rxo1* from maize specifically recognized the effector *AvrRxo1* from *Xanthomonas oryzae* pv. *oryzicola*, thereby triggering non-host resistance in maize. When *Rxo1* was introduced into rice, plants exhibited strong resistance to the *X. oryzae* pv. *oryzicola* (Zhao et al., 2005). After the pepper resistance gene *Bs2* was introduced into tomato, the transgenic tomato plants acquired stable resistance to *Xanthomonas campestris* pv. *vesicatoria* (Sendín et al., 2012). Similarly, transgenic expression of the *Arabidopsis* non-host resistance gene *EFR* in wheat enhanced resistance to the *Pseudomonas syringae* pv. *oryzae* (Schoonbeek et al., 2015). Given the similarity between aphid-plant interactions and pathogen-plant interactions, NHR is considered an innovative approach to developing broad-spectrum and durable aphid-resistant crops (Enders and Begcy, 2021). In this study, the identification of TaBBTI-1 as a defence component associated with poor-host resistance is particularly interesting because its insecticidal activity extended beyond cereal aphids to the chewing insect *H. armigera*. Future breeding programmes may therefore benefit from exploring naturally occurring non-host interactions as novel sources of insect resistance rather than focusing exclusively on resistant cultivars within wheat germplasm.

While non-host resistance provides a valuable resource of resistance, advances in molecular genetics have fundamentally changed how these resources are utilized. Molecular biological methods, including multi-omics analyses and molecular marker mapping, should be employed to identify resistance-related compounds and key regulatory genes, thereby elucidating the regulatory networks of wheat resistance to aphid. This study identified *TaAHL19* as a primary transcriptional regulator of flavonoid biosynthesis and aphid resistance through multi-omics analysis. Transcription factors have the potential to coordinate the entire defense pathway by simultaneously regulating multiple downstream genes. Recent advances in CRISPR/Cas-mediated genome editing provide unprecedented opportunities to fine-tune endogenous regulatory pathways without introducing foreign DNA (Chen et al., 2019). Editing promoter regions, modifying transcription factor binding sites or

precisely regulating inducible defence genes may represent promising strategies for enhancing aphid resistance while minimising undesirable effects on plant growth and yield. In addition, with the release and annotation of the wheat genome sequence (Rudi et al., 2018), the integration of GWAS with conventional breeding strategies enables rapid isolation of aphid-resistance genes. RNAi also provides an alternative strategy by directly targeting essential insect genes involved in feeding, development or virulence (Zotti et al., 2018; Christiaens et al., 2020). Salivary effectors have emerged as particularly attractive RNAi targets because they are required for successful manipulation of host immunity during aphid colonisation. Integrating RNAi technology with host genetic resistance may further improve the durability of aphid management by simultaneously targeting both plant defence and insect virulence mechanisms.

In this study, although the findings of this thesis provide several promising genetic and molecular resources for improving aphid resistance in wheat, the present work mainly focuses on understanding resistance mechanisms and providing functional evidence for the identified genes, metabolites, and salivary elicitors. Therefore, their potential application in resistance breeding or crop protection should be interpreted with caution. First, the resistance phenotypes associated with TaBBTI-1 and the TaAHL19–TaF3GT–rutin module should be further evaluated using stable genetic lines across different genetic backgrounds. Second, comprehensive agronomic assessments are needed to determine whether enhanced defense affects plant growth, yield, grain quality, or other important traits. Third, multi-year and multi-location field trials will be necessary to evaluate the stability of resistance under variable environmental conditions and different aphid population pressures. The ecological consequences of enhanced resistance, including potential effects on non-target organisms and interactions with natural enemies, should also be considered. Similarly, although HM6172 represents a valuable source of aphid resistance and Sa6007 provides insight into aphid-triggered plant defense, their practical value requires further genetic, agronomic, and ecological validation.

In conclusion, durable aphid-resistant breeding should integrate the continuous exploration of diverse resistance resources with advances in modern molecular biology. While extensive screening of wheat germplasm remains essential for identifying novel resistant phenotypes, multi-omics analyses and functional validation provide powerful approaches for dissecting the genetic architecture underlying aphid resistance and translating these traits into molecular breeding targets. Importantly, non-host resistance represents an underexploited source of broad-spectrum and durable defence mechanisms that could substantially broaden the genetic basis available for wheat pre-breeding. The integration of these complementary strategies will accelerate the development of wheat cultivars with more durable and sustainable resistance to aphids.

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List of publications

Accepted publications-first author:

1. **Liu, X.B.**, Wang, Q., Liu, Y., Zhang, Y., Francis, F., Chen, J.L. (2026). Plant-aphid interactions in cereal crops: wheat plant defense and aphid saliva-mediated coevolutionary arms race. *Insects*, 17, 672.
2. **Liu, X.B.**, Zhang, Y.M., Francis, F., Cheng, Y.M., Liu, Y.X., Chen, J.L., Zhang, Y. (2026). Induced Bowman-Birk trypsin inhibitor TaBBTI-1 in wheat during poor-host interactions with aphids confers broad-spectrum resistance to insect herbivores. *International Journal of Biological Macromolecules*, 340, 150204.
3. **Liu, X.B.**, Zhang, Y.M., Francis, F., Cheng, Y.M., Zhang, Y., Chen, J.L. (2024). Comprehensive aphid resistance evaluation of wheat varieties at seedling and adult stages in Huang-Huai-Hai region of China. *Journal of Plant Protection*, 51, 1427-1437. (in Chinese)
4. **Liu, X.B.**, Zhang, Y., Chen, J.L. (2023). Research progress on mechanism and genetics of aphid resistance in wheat crops. *Plant Protection*, 49, 181-189. (in Chinese)

Accepted publications-coauthor:

1. Zhang, Y., Fu, Y., **Liu, X.B.**, Francis, F., Fan, J., Liu, H., Wang, Q., Sun, Y., Zhang, Y.M., Chen, J.L. (2023) SmCSP4 from aphid saliva stimulates salicylic acid-mediated defence responses in wheat by interacting with transcription factor TaWKRY76. *Plant Biotechnology Journal*, 21, 2389-2407.
2. Fu, Y., **Liu, X.B.**, Wang, Q., Liu, H., Cheng, Y.M., Li, H.M., Zhang, Y., Chen, J.L. (2023) Two salivary proteins Sm10 and SmC002 from grain aphid *Sitobion miscanthi* modulate wheat defense and enhance aphid performance. *Frontiers in Plant Science*, 14.
3. Zhang, Y.M., **Liu, X.B.**, Sun, Y., Liu, Y., Zhang, Y., Ding, T.B., Chen, J.L. (2024) Salivary protein cyclin-dependent kinase-like from grain aphid *Sitobion avenae* suppresses wheat defense response and enhances aphid adaptation. *International Journal of Molecular Sciences*, 25, 4579.

Prepare to submit articles- first author:

1. **Liu, X.B.**, Wang, Q., Zhou, J.Y., Wu, P.P., Liu, H., Zhang, Y.M., Tan, X.L., Fan, J., Francis, F., Liu, Y., Zhan, Y.D., Liu, Y.X., Fu, Y., Chen, J.L., Zhang, Y. An AHL transcription factor enhances wheat resistance to aphids without compromising yield. Submitted to *Advanced Science* (Under review).

Communications:

1. **Liu, X.B.**, Wang, Q., Zhang, Y., Chen, J.L. (2023) Identification of wheat varieties' resistance to aphids and study on the resistance mechanism. In Beijing entomological society member day, Collection of papers, Beijing, China.
2. **Liu, X.B.**, Zhang, Y., Chen, J.L., Francis, F. (2025) Induced Bowman-Birk trypsin inhibitor TaBBTI in wheat during poor-host interactions confers resistance to aphids. In Second GxABT PhD Symposium, Poster, Gembloux, Belgium.

