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Identification of tomato leaf miner secretory proteins and their roles in influencing plant defenses

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ABSTRACT

The tomato leaf miner (*Tuta absoluta*) is a globally destructive pest that cause extensive damage to tomato crops by chewing mouthparts, leading to severe necrosis, fruit abortion, and substantial yield losses. To date, the elicitors/effectors of *T. absoluta* have not been characterized. In this study, we combined proteomic profiling of *T. absoluta*-infested tomato leaves with transcriptomic analysis of salivary glands to identify candidate molecules involved in herbivory-driven plant responses. Bioinformatics analyses predicted 40 candidate elicitors and effectors, which were subsequently assessed through transient expression assays in *Nicotiana benthamiana*. The results demonstrated that the candidate number 33 (*T. absoluta* 33, Ta33) induced cell death in both the intracellular space and the apoplast, while Ta21 triggered a strong apoplastic reactive oxygen species (ROS) burst. Conversely, Ta38 effectively suppressed INF1-induced cell death. Quantitative real-time PCR analysis further showed that these genes were highly expressed during the feeding stage, supporting their involvement in plant–insect molecular dialogue. This study systematically identified and characterized elicitors and effectors of *T. absoluta*, providing a foundational framework for elucidating its herbivory mechanisms and developing targeted management strategies.

1. Introduction

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a globally invasive pest originating from South America and has become a major quarantine threat across Europe, Africa, and Asia (Giorgini et al., 2019; Pandey et al., 2023). By 2023, *T. absoluta* had been documented in more than 100 countries or territories across five continents, with its distribution continuing to expand. This polyphagous insect feeds on over 50 plant species across 11 families, including Solanaceae, Cruciferae, and Legumes. Among these plants, tomatoes (*Solanum lycopersicum*) are particularly susceptible to damage caused by the tomato leaf miner. This insect, equipped with chewing mouthparts, cause severe damage by mining the mesophyll, feeding on fruit pulp, and attacking apical buds. Both open-field and greenhouse infestations can result in yield losses approaching 100 % in tomato crops (Abd El-Ghany and Faucheux, 2022; Acharya et al., 2023). Current management of the tomato leaf miner moth rely largely on conventional chemical pesticides; however, this approach is increasingly compromised due to resistance development, high application costs, and environmental concerns (D'Esposito et al., 2021). Understanding the interaction mechanisms between the tomato leaf miner and tomato plants provides the foundation for developing novel sustainable pest control strategies.

Previous studies have shown that insects can regulate plant defense responses by secreting two types of proteins into host plant cells via saliva, oral secretion (OS), and frass (Acevedo et al., 2015; Chen and Mao, 2020; Naalden et al., 2021). One class of these proteins is recognized by pattern recognition receptors located on the plant cell membrane, thereby activating plant pattern-triggered immunity (PTI), also referred to as herbivore-associated molecular patterns (HAMPs). PTI responses include Ca^{2+} fluxes, ROS bursts, activation of mitogen-activated protein kinase, and other downstream defenses (Snoeck et al., 2022). To date, approximately 30 elicitors have been identified, most of which are derived from insect saliva or oral secretions. For example, the saliva elicitors RP309 and RPH1 from *Riptortus*

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pedestris induce cell death and ROS bursts in plants (Su et al., 2019; Dong et al., 2022a). The cysteine protease Cathepsin B3 acts as an elicitor by interacting with an EDR1-like protein, triggering ROS production and ultimately suppressing aphid feeding (Guo et al., 2020). Similarly, chloroplast ATP synthase fragments present in the oral secretions of armyworm larvae are specifically recognized by receptor-like protein INR in legumes, leading to the induction of defense signals such as ROS and ethylene (Steinbrenner et al., 2020).

The other class of secretory proteins, known as effectors, employ diverse strategies to suppress host defense mechanisms and facilitate insect feeding. Some effectors are recognized by plant resistance proteins, triggering a more robust immune response, such as hypersensitive response (HR)-like cell death – a process termed effector-triggered immunity (ETI) (Yuan et al., 2021; Ngou et al., 2022). Conversely, other effectors evade host recognition, thereby promoting insect feeding behavior. Many effectors directly target components of the PTI pathway. For instance, *Bemisia tabaci* Btfer1 (Su et al., 2019) and *Apolygus lucorum* Al6 inhibit INF1-induced cell death and the ROS-mediated immunity pathway (Dong et al., 2020). Further research has shown that effectors can suppress plant immunity by interacting with specific target proteins within the host plant. The nutritional protein vitellogenin of the brown planthopper functions as an effector by interacting with the rice transcription factor OsWRKY71, thereby weakening hydrogen peroxide-mediated plant defense (Ji et al., 2021). Similarly, the salivary proteins Al106 from *A. lucorum* inhibits the E3 ubiquitin ligase activity of PUB33, resulting in suppression of PTI (Dong et al., 2022b). In addition, some effectors modulate plant hormonal pathways. For example, the cotton bollworm effectors HARP1 and HAS1 proteins block jasmonate (JA) signaling through dual inhibition (Chen et al., 2019, 2023).

In recent years, advances in salivary gland transcriptome analysis combined with proteomic techniques have facilitated the identification of salivary components in diverse insect species (Wu et al., 2021). This has improved our understanding of the functional properties of saliva proteins during feeding. Studies have shown that salivary components from many piercing-sucking insects, such as aphids and planthoppers, are critical for successful feeding on host plants (Huang et al., 2023). However, research on the oral secretions of chewing insects remains limited, with only a few studies conducted in Lepidoptera-and notably, none involving the tomato leaf miner (Rivera-Vega et al., 2017; Wang et al., 2023).

Systematic identification of insect elicitors and effectors is pivotal for unraveling the molecular mechanisms underlying insect-host interactions and for developing innovative pest management strategies (Wang et al., 2023). However, whether analogous molecular players-such as elicitors or effectors-exist in the tomato leaf miner remains unknown. To address this gap, we employed a multi-omics approach integrating proteomic profiling of tomato leaves infested by *T. absoluta* with salivary gland transcriptomic datasets. By combining these datasets and performing bioinformatics analysis, we predicted 40 candidate elicitors or effectors. Functional assays assessing their effects on plant defense responses-such as induction of cell death, generation of ROS burst, and suppression of cell death-led to the identification of three candidates (Ta21, Ta33, and Ta38). These three genes were highly expressed during the feeding stages, supporting their roles in mediating the interaction between *T. absoluta* and its host plants.

2. Materials and methods

2.1. Insects and plants

T. absoluta colonies were maintained on tomato plants in a climate-controlled chamber set to 25 ± 2 °C, with 50–60 % relative humidity and a 10 h: 14 h light: dark photoperiod. *Nicotiana benthamiana* and *Solanum lycopersicum* (var. Mico-Tom) were cultivated separately under but with16-h light and 8-h dark cycle. All experimental plants were grown from seed in 500 cm^3 pots filled with a commercial potting substrate.

2.2. Plant treatment

Uniform, healthy tomato seedlings (5–20 days old) were individually transplanted into separate cages. Third-instar *T. absoluta* larvae were starved for 2 h before being introduced individually onto leaves of the experimental plants (one larva per leaf). Control plants were maintained without larval exposure. After a 36-h feeding period, the larvae were removed. Leaf tissues extending 1 cm outward from the feeding sites were excised using dissecting scissors, immediately wrapped in aluminum foil, and snap-frozen in liquid nitrogen for preservation. Corresponding leaf regions from untreated control plants (of the same plant lineage and leaf position) were harvested simultaneously. At least ten biological replicates (leaves) were collected per condition to ensure statistical reliability.

2.3. Protein extraction and digestion

Tomato leaf samples were collected from two experimental groups: healthy leaves and leaves infested by *T. absoluta*. For protein extraction, approximately 1 g of frozen leaf tissue was ground into a fine powder under liquid nitrogen. The powder was then homogenized in SDT buffer (4 % SDS, 100 mM Tris-HCl, 1 mM DTT, pH 7.6) supplemented with a protease inhibitor cocktail. After incubation at 95 °C for 5min, the lysates were clarified by centrifugation and the supernatant was collected. Protein concentration was determined using a BCA Protein Assay Kit (Bio-Rad, USA). For in-solution digestion, equal amounts of protein from each sample were subjected to trypsin digestion following the filter-aided sample preparation (FASP) protocol. Briefly, proteins were reduced, alkylated, and digested overnight with sequencing-grade trypsin at 37 °C. The resulting peptides were desalted using C18 solid-phase extraction cartridges (Empore™ SPE Cartridges C18, 7 mm I.D., 3 mL, Sigma), concentrated by vacuum centrifugation, and reconstituted in 40 μL of 0.1 % formic acid for LC-MS/MS analysis.

2.4. LC-MS/MS analysis

Peptide samples were analyzed using a timsTOF Pro mass spectrometer coupled to a nanoElute ultra-high-performance liquid chromatography (UHPLC) system (Bruker Daltonics, Germany). Peptides were loaded onto a homemade analytical column (C18, 25 cm length × 75 μm inner diameter, 1.9 μm particle size) and separated using a 45-min linear gradient of buffer B (99.9 % acetonitrile with 0.1 % formic acid) in buffer A (0.1 % formic acid in water) at a constant flow rate of 300 nL/min. The mass spectrometer was operated in positive ion mode and configured in parallel accumulation–serial fragmentation (PASEF) mode. Full ion mobility-resolved MS spectra were acquired over an m/z range of 100–1700 and an ion mobility range ($1/K_0$) of 0.75–1.35 Vs/cm^2. Each duty cycle consisted of one MS survey scan followed by ten PASEF MS/MS scans. The target intensity was set to 1.5 k with a minimum intensity threshold of 2500. Dynamic exclusion was enabled with a release time of 0.4 min to minimize redundant fragmentation of previously sequenced precursors.

2.5. Identification and quantitation of proteins

Raw MS data were processed using MaxQuant (version 2.5.2.0) with the integrated Andromeda search engine. Tandem mass spectra were searched against the *T. absoluta* protein database. Carbamidomethylation of cysteine was specified as a fixed modification, whereas methionine oxidation and N-terminal acetylation were considered variable modifications. A false discovery rate (FDR) was controlled at 1 % at both the peptide and protein levels. Protein quantification was carried out using the label-free quantification (LFQ) algorithm integrated in

MaxQuant.

2.6. Dissection of salivary glands and RNA extraction

Salivary glands were dissected from 3rd – 4th larva of *T. absoluta* in the 1x phosphate-buffered saline (1x PBS) using fine forceps. The isolated glands were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from 100 salivary glands using the PureLink RNA Mini Kit (Thermo Fisher, USA) according to the product manual. RNA integrity and quality were assessed using a NanoDrop™ spectrophotometer and an Agilent Bioanalyzer.

2.7. RNA sequencing and transcriptome analysis

Whole-body samples: Healthy 3rd – 4th larva of *T. absoluta* were randomly collected. Five intact insects (without dissection) were pooled as one biological replicate to represent the global transcriptional background. Salivary gland samples: Salivary glands from 100 insects were pooled as one biological replicate. For each sample type, three independent biological replicates were prepared. RNA sequencing was performed using the Illumina NovaSeq 6000 platform using paired-end 150 bp reads. Raw sequencing data were processed using fastp (v0.23.2) to remove low-quality reads (Phred score <30), adapter contamination, and reads with uncertain base calls (Chen et al., 2018). Clean reads were aligned to the *T. absoluta* reference genome (GCA_029230345.1) using HISAT2 (v2.2.1) (Kim et al., 2019; Liu et al., 2023), followed by transcript assembly with StringTie (v1.3.0) (Pertea et al., 2015). Gene expression levels were quantified as fragments per kilobase of transcript per million mapped reads (FPKM). Differential gene expression analysis was performed using DESeq2 (v1.46.0) to identify significant genes with adjusted p-values <0.05 and a $\log_2$ fold change greater than 1 (Love et al., 2014).

2.8. Bioinformatics analysis

Initial screening of candidate secreted proteins was performed using a combination of bioinformatic tools. The presence and cleavage site of N-terminal signal peptides were predicted using SignalP v.5.0 (http://www.cbs.dtu.dk/services/SignalP/) (Almagro Armenteros et al., 2019). To filter out proteins likely to be retained in cellular membranes, candidates were analyzed for transmembrane helices using TMHMM v.2.0 (Krogh et al., 2001). Finally, the functional domain architecture of each candidate was determined via a search against the Pfam-A database (release 35.0) using the online HMMER search service (E-value threshold < 1e-5).

2.9. Plasmid construction

For transient expression in *N. benthamiana*, candidate genes were amplified from the complementary DNA (cDNA) of *T. absoluta* larva using gene-specific primers and inserted into the plant expression vector pBin-3HA (a plasmid vector carrying HA tag) using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) (Dong et al., 2022a; Lu et al., 2023). Individual colonies of each construct were verified by PCR detection and sequencing. All primers were synthesized by Sangon Biotech (Shanghai, China) and the primers used in this study are listed in Table S4.

2.10. Quantitative real-time polymerase chain reaction analysis

cDNA of *T. absoluta* was synthesized from total RNA using the HiScript II QRT SuperMix Kit, and Quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix (Vazyme) on a QIAquant 96 2Plex under the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 61 °C for 30 s. A melting curve analysis was then conducted with the following program: 94 °C for 15 s, 64 °C for 1 min, and 94 °C for 15 s. The relative expression levels of genes were normalized to actin (Genebank: MZ054824.1) as the reference gene.

2.11. Agrobacterium tumefaciens infiltration assay

The correctly sequenced recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *A. tumefaciens* cells were collected, re-suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES pH 5.7, 150 μM acetosyringone) and adjusted to an optical density at 600 nm (OD_{600}) of 0.4–0.6. Agroinfiltration was performed on approximately 20-day-old *N. benthamiana* plants using a 1 mL syringe (Liu et al., 2011; Lu et al., 2023).

2.12. Oxidative burst assay

The oxidative burst assay was carried out as previously described (Zhou et al., 2024). Candidate proteins were expressed in *N. benthamiana* leaves for 24–36 h and then immersed in 1 mg/ml DAB-HCl solution (pH 2.8, Sigma-Aldrich, USA) at 25 °C for 6–8 h, finally decolorized in boiling 95 % (v/v) ethanol. Stained leaves were photographed under natural light using a Canon EOS 250D camera.

2.13. Western blot analysis

N. benthamiana leaves transiently expressing the specified protein for 48 h were frozen in liquid nitrogen and ground into a fine powder. The powder was resuspended in extraction buffer (50 mM HEPES, 150 mM KCl, 1 mM EDTA, and 10 % Triton X-100; pH 7.5) supplemented with 1 mM DTT and 1 × protease inhibitor cocktail (Sigma-Aldrich), and then centrifuged at 13,000 rpm for 15 min at 4 °C. The proteins were separated by 12 % SDS-PAGE and transferred onto a PVDF membrane using a rapid wet-transfer system (GenScript, eBlot™ L1, China). The membrane was then blocked with 5 % non-fat dry milk in TBST for 1 h, washed three times with TBST (5 min each), and incubated with anti-HA-HRP (1:5000; MBL, M180-7) for 2 h. Signals were detected using ECL substrates (Vazyme) and imaged with an automated chemiluminescence imaging system. The membrane was stained with Ponceau S solution to verify protein transfer.

3. Results

3.1. Identification of T. absoluta proteins in infested tomato leaves

To investigate the presence of insect-derived proteins in response to insect herbivory, we performed a proteomic analysis of tomato leaf samples collected under two conditions: healthy (uninfested) leaves and leaves infested by *T. absoluta*. Across all samples, a total of 603 *T. absoluta* proteins were identified across all samples. Strikingly, 351 of these proteins were specifically detected in infested leaf samples, indicating their origin from insect feeding activity or salivary secretions (Fig. 1, Table S1). These findings indicate the active transfer of insect protein into host tissues during infestation and highlight potential candidate effectors involved in plant–insect interactions.

3.2. Transcriptomic profiling of salivary glands reveals tissue-specific gene expression patterns

To investigate tissue-specific gene expression in *T. absoluta*, we compared the transcriptomes of salivary glands and whole-body tissues. Our analysis revealed 2182 genes that were significantly upregulated in salivary gland samples compared with whole-body samples, suggesting their specific transcriptional activity may be associated with feeding and host interaction (Fig. 2, Table S2). By integrating transcriptomic and proteomic data, we further identified 80 genes that were not only transcriptionally upregulated but also detected at the protein level in salivary gland proteome (Figs. 1 and 3), supporting their biological

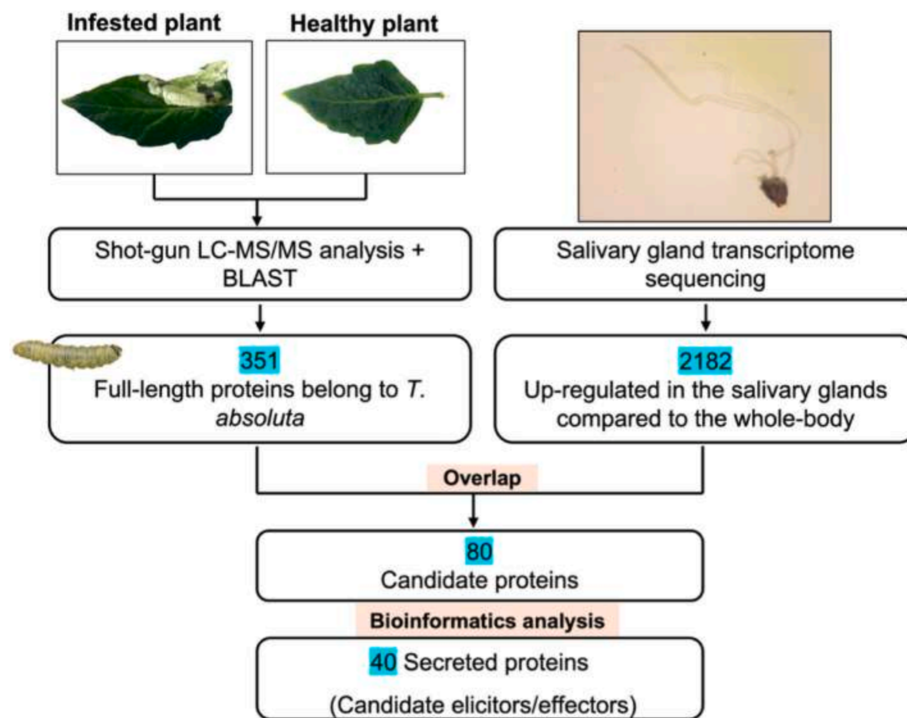

Fig. 1. Workflow and overview for identifying secreted proteins of *Tuta absoluta*.
The infested leaves by *T. absoluta* were collected for shotgun LC-MS/MS analysis and salivary glands were dissected from the 3rd-4th instar larvae for transcriptome analysis. Bioinformatics analysis of combined datasets yielded 40 candidate elicitors and effectors.

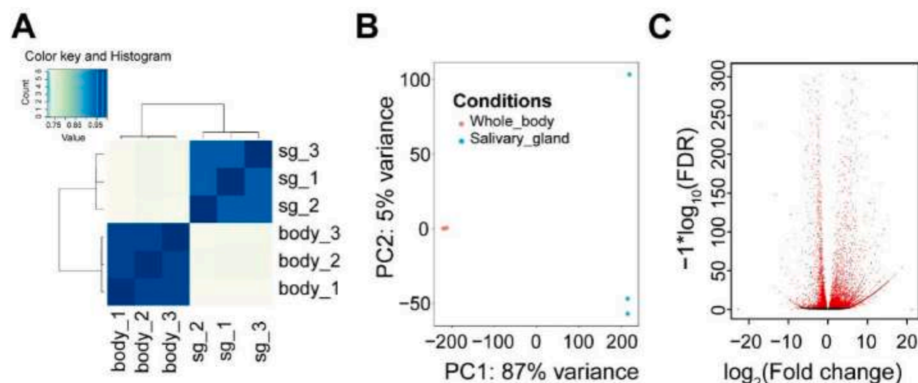

Fig. 2. Differential gene expression between salivary gland and whole-body tissues in *Tuta absoluta*.
(A) Pearson correlation analysis among biological replicates, showing overall sample consistency. (B) Principal component analysis (PCA) demonstrating clear separation between salivary gland and whole-body transcriptomes. (C) Volcano plot illustrating differentially expressed genes between the two tissue types; significantly upregulated and downregulated genes are highlighted.

relevance during herbivory.

Effector proteins from insects and plant pathogens often share common structural features, such as the presence of an N-terminal signal peptide for secretion and a relatively small protein size. Guided by these features, we used SignalP v5.0 and TMHMM 2.0 programs to screen the 80 overlapping proteins for those carrying a signal peptide but lacking transmembrane domain (Fig. 1). This screening yielded 40 candidates (Table S3), representing a set of high-confidence salivary effectors that may play crucial roles in mediating interactions between *T. absoluta* and its host plant.

3.3. Screening of secreted proteins that induce cell death

Cell death is an important indicator of plant defense responses triggered by elicitors and effectors. To identify potential elicitors or effectors from *T. absoluta*, we employed an *Agrobacterium*-mediated transient expression system in *N. benthamiana* to examine cell death phenotypes. Following the method described in Dong et al. (2022a), we cloned both the full-length and signal peptide–deleted versions of 40 candidate effector genes from *T. absoluta* cDNA and assembled them into plant expression vectors for expression in *N. benthamiana*. Green fluorescent protein (GFP) was used as a negative control, whereas the *Phytophthora infestans* elicitor INF1 served as a positive control (Domazakis et al., 2018; Z. Chen et al., 2023) (Fig. 4A–D). Among all tested candidates, Ta33 was the only protein that induced visible cell death, and this phenotype occurred in both intracellular (Ta33, without signal peptide) and the apoplastic (Ta33 + sp, with signal peptide). In contrast, none of the other candidate genes triggered cell death symptoms (Fig. 4C and D, Fig. S1, Fig. S2). Western blot analysis confirmed successful expression of all recombinant proteins in *N. benthamiana* (Fig. S3). Moreover, the

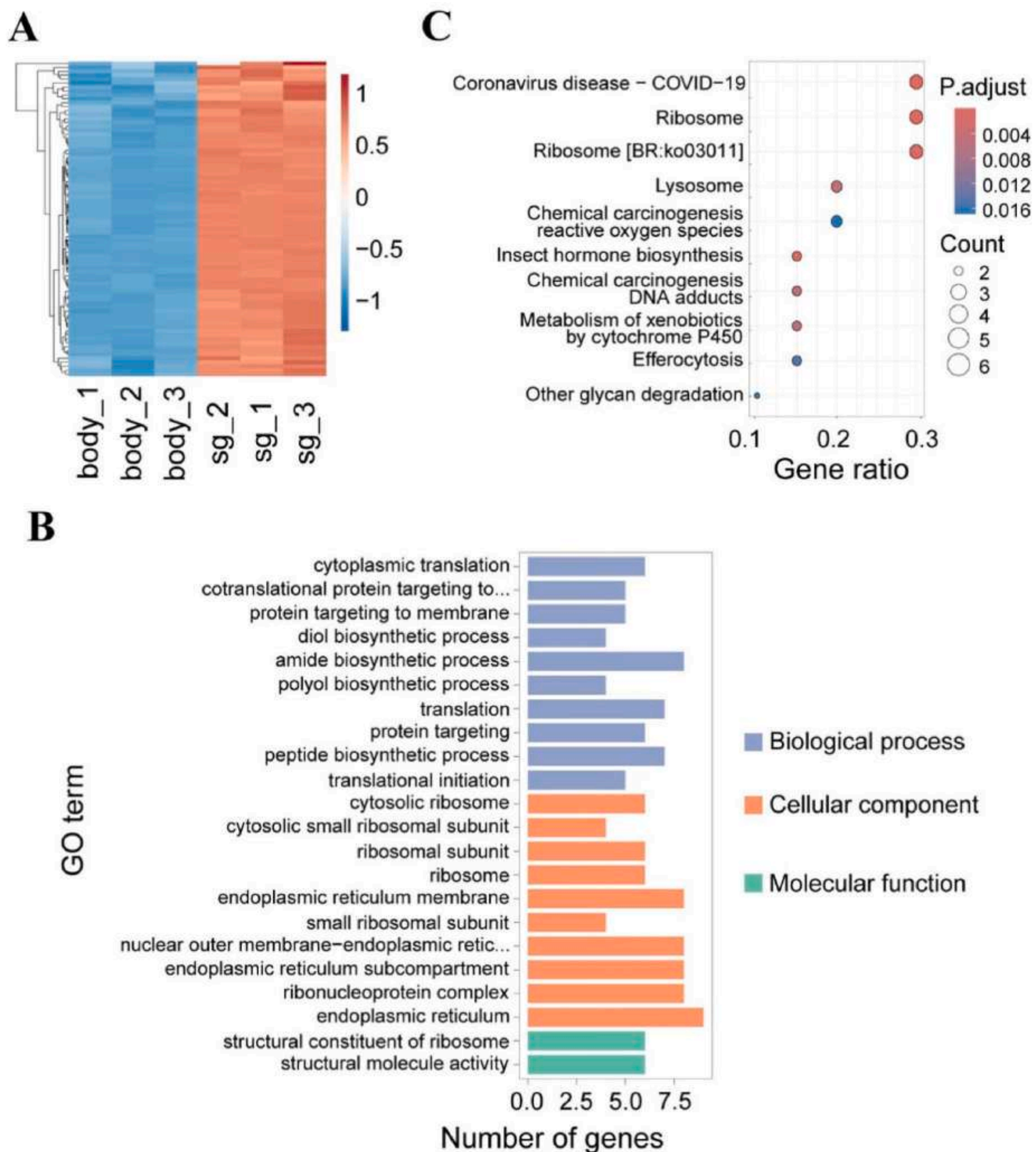

Fig. 3. Functional and expression analysis of 80 overlapping gene–protein candidates in *Tuta absoluta* salivary glands.
(A) Heatmap of normalized gene expression levels across salivary gland and whole-body samples. (B) GO enrichment analysis showing significantly enriched biological processes, molecular functions, and cellular components among the 80 genes. (C) KEGG pathway enrichment analysis highlighting major pathways associated with salivary gland-specific gene expression.

cell death phenotype induced by Ta33 was consistently observed in all fifteen biological replicates, validating the robustness of the response (Fig. 4E and F). Taken together, these results indicate that Ta33 is a potential elicitor or avirulence (Avr) effector, suggesting its involvement in *T. absoluta*-tomato interactions.

3.4. Screening of secreted proteins that trigger ROS burst

Reactive oxygen species (ROS) are central to plant immune responses, serving as both direct antimicrobial agents and as signaling molecules that activate defense pathways against insects and pathogens (Qi et al., 2017). To further screen candidate elicitors or effectors, we examined ROS accumulation using DAB staining. As expected, strong reddish-brown precipitates were observed in INF1-overexpressing leaf regions (positive control), confirming a robust ROS burst. Similarly, reddish-brown precipitates were detected in leaf regions overexpressing Ta33, Ta33 (+sp) and Ta21 (+sp) (Fig. 5A–D, Fig. S4, Fig. S5). These findings support the notion that Ta33 functions as a potential elicitor or Avr effector. In addition, the apoplast-specific ROS burst induced by Ta21 (+sp) suggests that this protein may be recognized by plasma membrane pattern-recognition receptors, consistent with the activity of a HAMP-like elicitor.

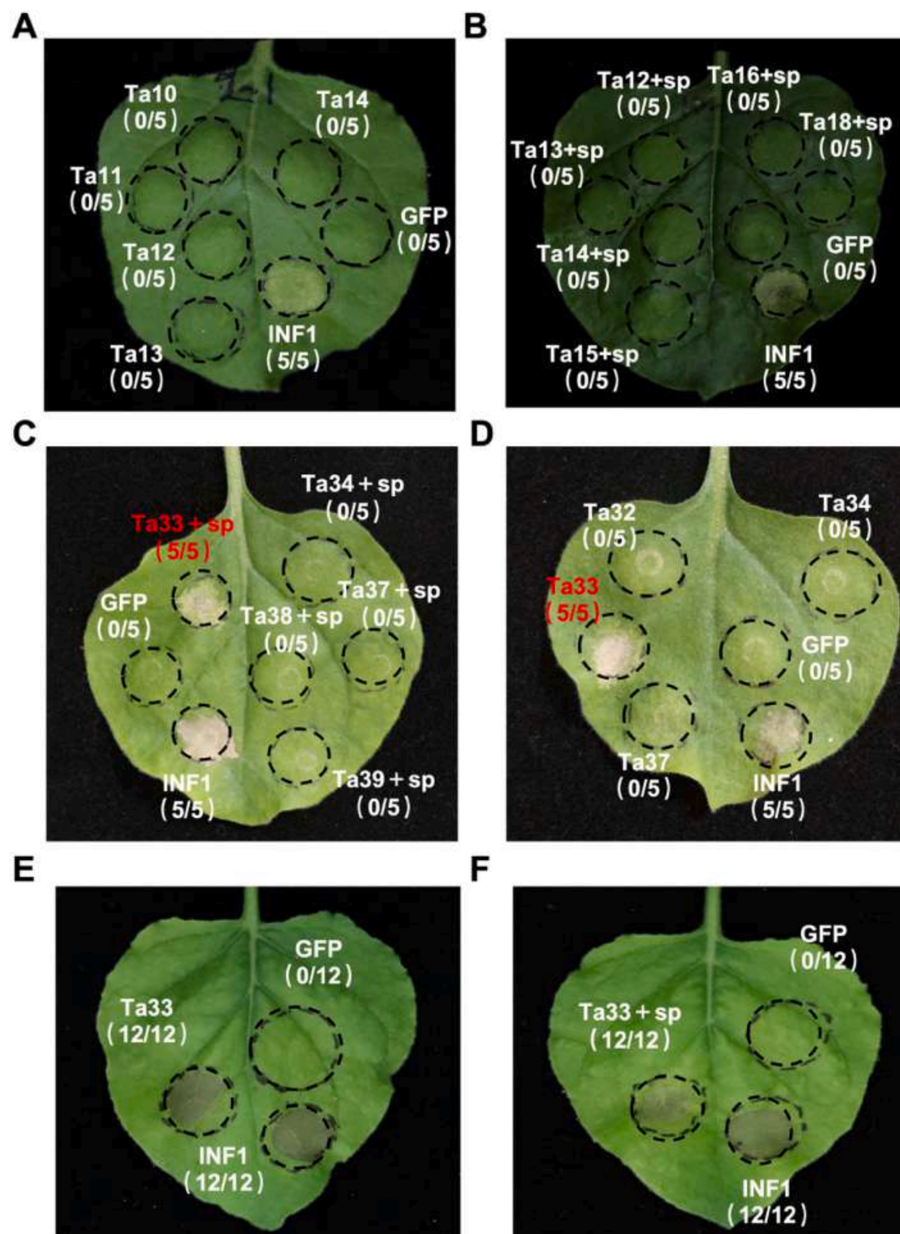

Fig. 4. Screening of *Tuta absoluta* candidate elicitors/effectors that induce cell death.
(A–D) Candidate elicitors/effectors, the cell death-inducing gene INF1, and empty control (GFP) were expressed in *Nicotiana benthamiana* leaves via agroinfiltration (Representative pictures). GFP is used as the negative control, INF1 is the positive control that induced cell death. (E–F) Ta33 and Ta33 (+sp) induce cell death in *N. benthamiana* leaves. The leaves were photographed at 4 days after agroinfiltration. The ratios in the circles represent the number of samples inducing cell deaths to the total number of experiments. SP, signal peptide.

3.5. Screening of secreted proteins that suppress cell death

The ability to suppress INF1-mediated cell death is a widely used criterion for initially screening insect effectors that suppress defense-associated cell death, enabling the identification of candidates with immune-attenuating functions (Wang et al., 2011; Dong et al., 2020; Zhang et al., 2024). To identify insect effectors that can suppress INF1-induced defense-associated cell death, a total of 40 effector genes were screened in *N. benthamiana* through an *Agrobacterium*-mediated transient expression assay. *Agrobacterium* strains carrying each effector gene were infiltrated into *N. benthamiana* leaves either simultaneously with a strain carrying the INF1 gene or 24 h in advance. Al106 from mirid bugs, known to inhibit INF1-triggered cell death, severed as a positive control (Dong et al., 2022b). Among all tested candidates, only Ta38 and the positive control Al106 were able to suppress INF1-triggered cell death, whereas GFP and other candidate effectors failed to inhibit cell death (Fig. 6A–D, Fig. S6). Subsequent assays confirmed that Ta38 consistently blocked INF1-induced cell death across fifteen biological replicates, demonstrating the robustness of this phenotype (Fig. 6A–D). Together, these results identify Ta38 as a potential effector that inhibits plant defense response during *T. absoluta* herbivory.

3.6. Transcription expression patterns of candidate proteins in different developmental stages and tissues

To further characterize the candidate elicitors and effectors, we analyzed their expression patterns across different developmental stages and tissues of the tomato leaf miner. The results showed that the transcriptional abundance of *Ta21*, *Ta33* and *Ta38* was significantly

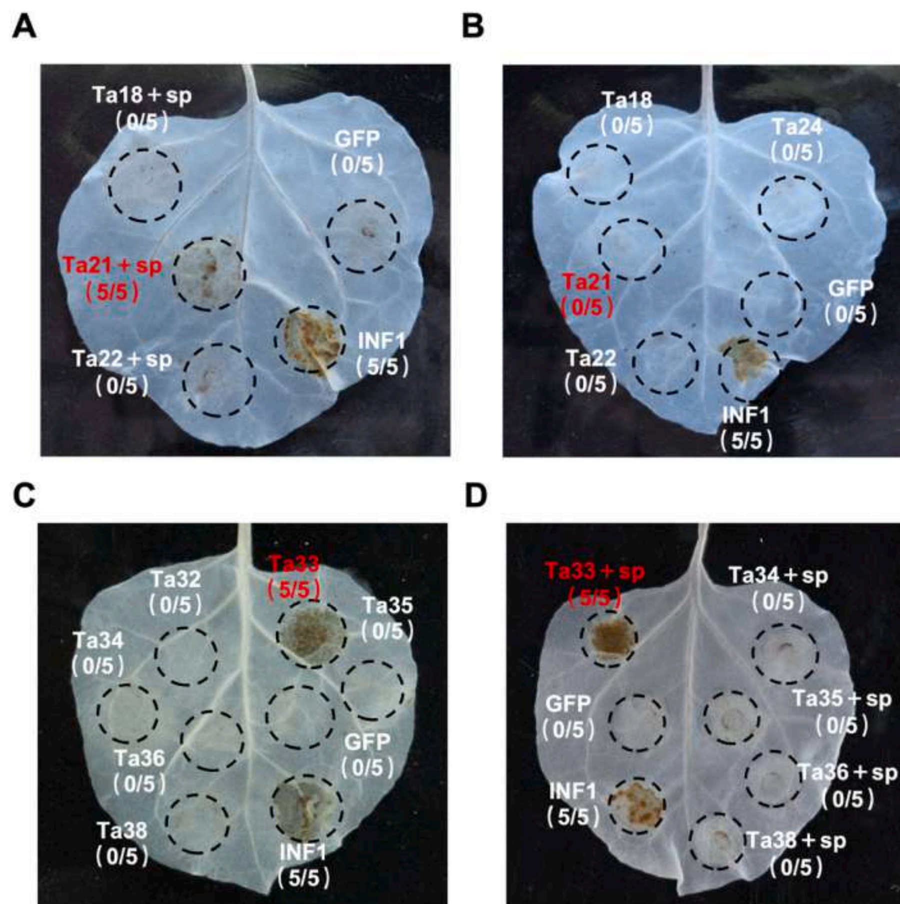

Fig. 5. Screening of *Tuta absoluta* candidate elicitors/effectors that induce ROS burst.
(A–D) ROS accumulation associated with each candidate elicitors/effectors (Representative pictures). DAB staining was performed at 2 d after infiltration on leaves containing candidate elicitors/effectors, GFP and INF1. Ta21 (+sp) induce ROS burst in *N. benthamiana* leaves (A), but Ta21 failed (B). Ta33 (+sp) and Ta33 induce ROS burst in *N. benthamiana* leaves (C, D). Five biological repeats were performed for each candidate elicitors/effectors.

elevated during plant-feeding larval stages (1st to 4th larva), compared with non-feeding life stages such as eggs, pupae, and adults (Fig. 7A–C, E). We also detected the relative transcription of *Ta21*, *Ta38,* and *Ta33* in different body tissues, including the salivary gland (Sg), fat body (Fat) and gut. QPCR analysis revealed that all three genes were strongly enriched in salivary glands, showing 3- to 100-fold higher expression relative to other tissues (Fig. 7B–D, F). This expression profile is consistent with salivary gland transcriptome data. Collectively, these results highlight that *Ta21*, *Ta38*, and *Ta33* are strongly associated with feeding-related processes. Their stage- and tissue-specific expression profiles suggest potential roles in mediating the molecular dialogue between *T. absoluta* and its host plants, including herbivore recognition and modulation of plant defense signaling pathways.

4. Discussion

Insect elicitors and effectors are central players in the co-evolutionary arms race between plants and herbivores, providing valuable insights into the molecular basis of insect feeding behaviors and plant defense against herbivory. In this study, proteomic analysis identified 350 *T. absoluta* proteins secreted into tomato leaves during feeding, while transcriptomic profiling revealed 2182 genes highly expressed in the salivary glands. By integrating these datasets, we narrowed the list down to 40 candidate elicitor/effector genes. Using a combination of bioinformatic prediction and transient expression assays in *N. benthamiana*, we systematically characterized these candidates. Among them, Ta33 was found to induce HR-like cell death and ROS burst, while Ta21 acted as a putative elicitor of ROS accumulation. In contrast, Ta38 functioned as an immune-suppressing effector that inhibited INF1-triggered cell death. Expression analyses further demonstrated that *Ta21*, *Ta33*, and *Ta38* were strongly upregulated in the salivary glands and during larval feeding stages, highlighting their potential roles in mediating the molecular dialogue between *T. absoluta* and its host plant.

Previous approaches for predicting insect elicitors and effectors have primarily relied on salivary gland transcriptome sequencing and salivary protein identification, as exemplified by studies on *Riptortus pedestris* (Dong et al., 2022a) and *Cnaphalocrocis medinalis* (Cui et al., 2024). However, a key limitation of these approaches is their inability to directly detect proteins that are actually secreted into host tissues or to systematically characterize bioactive molecules present in insect exudates such as honeydew and haemolymph (Wang et al., 2023). Here, we addressed this limitation by employing a targeted proteomic approach to directly profile proteins deposited in *T. absoluta*-infested tomato leaves (Fig. 1). This strategy improved the accuracy of effector prediction by reducing reliance on transcriptomic inference alone. Although our analysis focused on the intersection between salivary gland transcriptomic and proteomic datasets, it is noteworthy that the non-overlapping candidates also represent valuable resources for future effector discovery. Together, our findings not only validate current bioinformatic pipelines for effector prediction but also provide a critical framework to guide the refinement and integration of future methodologies aimed at elucidating herbivore-derived elicitors and effectors.

Insect-derived elicitors are typically perceived by plant plasma

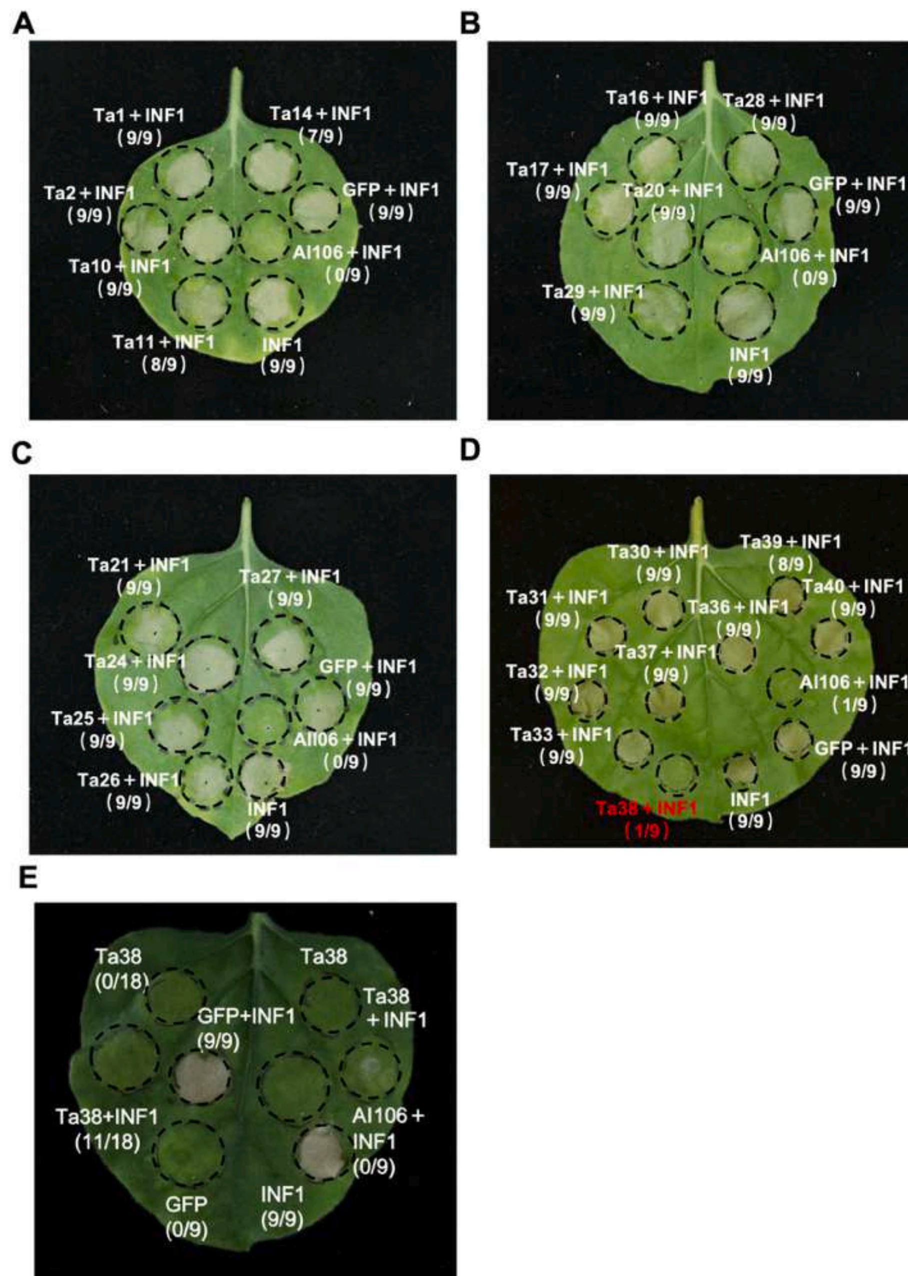

Fig. 6. Screening of *Tuta absoluta* candidate effectors that inhibit cell death.
(A–D) *N. benthamiana* leaves were first infiltrated with recombinant strains of *A. tumefaciens* carrying candidate effector genes, GFP or Al106 and INF1 was injected in the same region after 24 h Ta38 inhibits INF1-triggered cell death (D–E). The leaves were photographed 5 d after agroinfiltration and nine biological repeats were performed for each candidate effector. The ratios in the circles represent the number of samples inducing cell deaths to the total number of experiments.

membrane pattern-recognition receptors (Wang et al., 2023), initiating defense responses such as cell death and ROS burst. Previous studies have identified several such elicitors—for example, Nlsp5 from the brown planthopper relies on the plasma membrane pattern-recognition co-receptors BAK1 to trigger necrosis and oxidative bursts (Qi et al., 2025), and the HAMP RPH1 from *Riptortus pedestris* triggers typical PTI responses (Zhou et al., 2024). These findings provide a conceptual framework for interpreting elicitor activity in herbivorous insects. In our study, heterologous overexpression of Ta33 in the apoplast of *N. benthamiana* elicited both cell death and ROS burst consistent with HAMP (Fig. 5). Unexpectedly, intracellular expression of Ta33 also induced strong HR-like cell death, a response typically associated with Avr effectors recognized by intracellular immune receptors. This dual behavior suggests that Ta33 may have a more complex mode of action than canonical insect elicitors, potentially involving multiple host recognition pathways or spatially dynamic interactions within plant tissues. Strategies such as fluorescent protein tagging, and effector-host protein interaction screens will be essential to resolve these possibilities. In contrast, only apoplastic expression of Ta21 (Ta21+sp) in *N. benthamiana* elicited a ROS burst, whereas intracellular expression of Ta21 did not (Fig. 5). This phenotype conforms to the properties of an extracellular elicitor. Subsequent studies should aim to identify the active region of Ta21 and its host receptor, which may provide novel molecular targets for the development of next-generation pest control strategies. Finally, our findings underscore a methodological consideration: expressing candidates with and without signal peptides provides a useful first approximation of apoplastic versus intracellular function, signal peptide fusion does not unequivocally ensure extracellular

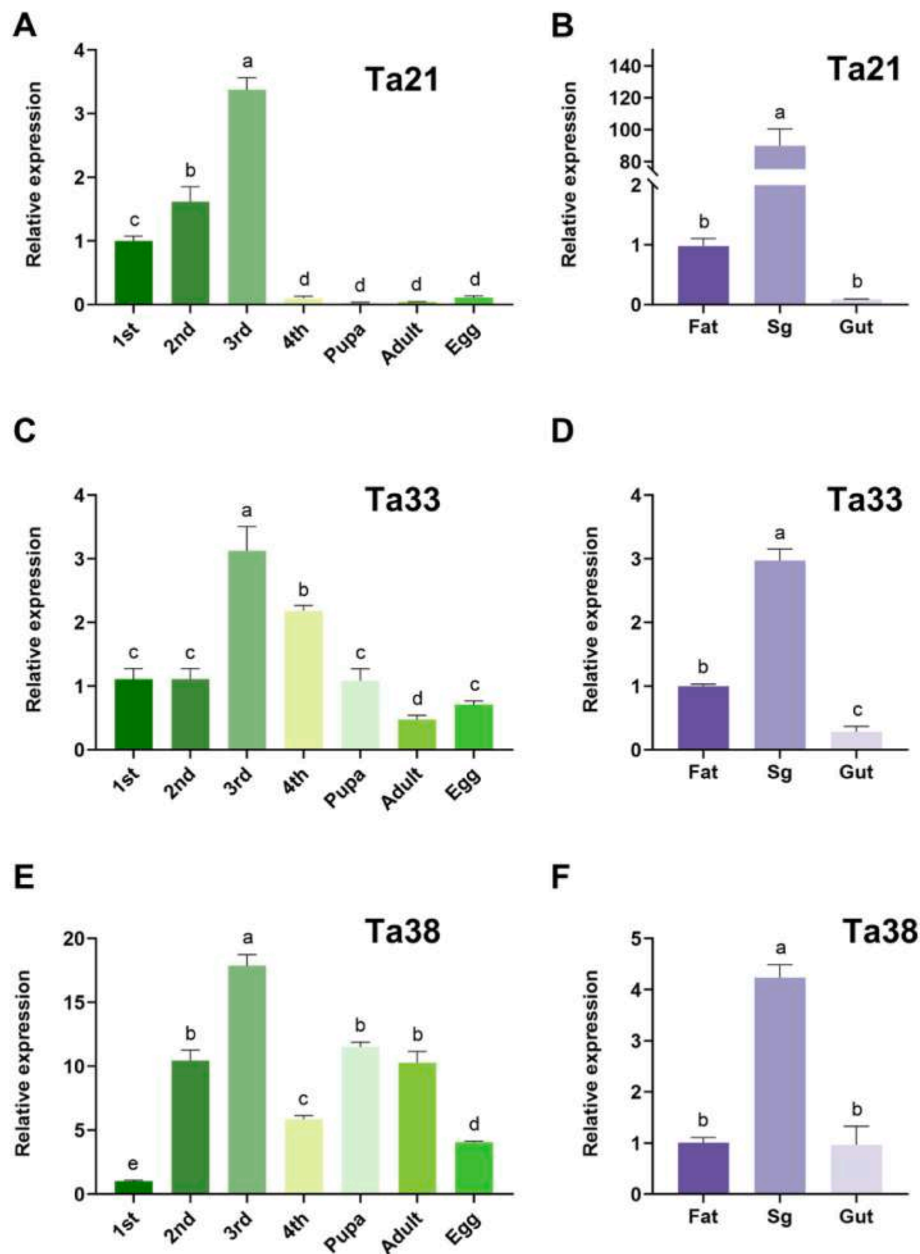

Fig. 7. Analysis of the expression pattern of *Ta21*, *Ta33*, and *Ta38*.
(A, C, E) Relative expression levels of *Ta21*, *Ta33* and *Ta38* at different development stages (egg, first to fourth larva, pupa and adult). (B, D, F) Relative expression patterns of *Ta21*, *Ta33, and Ta38* in different tissues (Sg, salivary gland; Fat, fat body; Gut). Data were normalized against *β-actin* gene expression, and the bars represent means (±SD) of three repeats. Different letters above the bars indicate significant differences, as determined by Tukey's honestly significant difference test ($P < 0.05$).

targeting (Dou et al., 2008; Oh et al., 2009). More rigorous localization validation will therefore be necessary for key candidates. Functional secretion assays (Jacobs et al., 1997; Yin et al., 2021), plasmolysis-based localization (Yin et al., 2021), and replacement of native signal peptides with well-characterized ones (e.g., PR1) (Song et al., 2021) represent critical next steps to refine localization-dependent interpretations of elicitor and effector activity.

Insects deploy diverse effector proteins to interfere with plant defense pathways, employing strategies such as inhibition of defense signaling, mimicry, hijacking, and subversion (Fu et al., 2025; Cai et al., 2025). These manipulations facilitate nutrient acquisition and promote successful colonization of the host. Well-characterized examples include the *Nilaparvata lugens* effector Nl14 which mimics a host immune regulator to facilitate infestation (Fu et al., 2025), and the cotton bollworm effector PPI5 which targets FKBP17-2 to inhibit endoplasmic reticulum immunity and JA/SA responses, enhancing insect feeding activity (Wang et al., 2024). In contrast to effectors that suppress immunity, elicitor such as INF1 activate PTI in plants. Because INF1 reliably induces cell death, the ability of to suppress INF1-induced cell death has become a widely accepted hallmark of PTI suppression and an established criterion for effector screening (Wang et al., 2011; He et al., 2018). This approach has led to the identification of several effectors, including the *Phytophthora sojae* RXLR effectors Avh52 and Avh331, the *Magnaporthe oryzae* effector Nls1 (Irieda et al., 2019), and the *A. lucorum* effectors Al6 and Al106 (Dong et al., 2020). In this study, our identification of Ta38 as a suppressor of INF1-induced cell death suggests that *T. absoluta* deploys immune-attenuating effectors analogous to those characterized in other herbivores and pathogen. Transcriptomic and

qPCR analyses further confirmed that Ta38 is highly expressed in the salivary glands, indicating its secretion into host plants via oral secretions—a feature shared with other reported effectors such as BtmiR29-b (Han et al., 2025), PPI5 (Wang et al., 2024), and Al106 (Dong et al., 2022b). Interestingly, *Ta38* was also expressed in the pupal and the adult stages (Fig. 7E), suggesting a potential additional role in the growth and development of the tomato leaf miner. Future research efforts should prioritize elucidating the molecular mechanisms by which Ta38 suppresses plant defense responses, concomitantly investigating its potential role in regulating insect developmental processes. Such studies would provide critical insights into the molecular basis of the invasion strategies employed by *T. absoluta.*

In our study, the functional screening of candidate elicitors/effectors was conducted by using an *Agrobacterium*-mediated transient expression system in *N. benthamiana* following well-established methodologies (Dong et al., 2022a; Rao et al., 2019). However, as *T. absoluta* naturally infests tomato and insects often exhibit host-specific adaptability, effector functionality may differ across plant species (Fernandez de Bobadilla et al., 2022). Classic examples illustrate this specificity, glucose oxidase from the American *Helicoverpa zea* suppresses nicotine production and immunity in tobacco (Musser et al., 2002, 2005), yet acts as an inducer of JA accumulation and late immune responses in tomato (Tian et al., 2012). Likewise, the potato aphid effector Me47 elicits the PTI response in tomato but not in *Arabidopsis thaliana* (Kettles and Kaloshian, 2016). Thus, although tomato and *N. benthamiana* are phylogenetically related Solanaceae species—and although *T. absoluta* has been documented as a potential threat to tobacco (Zhang et al., 2022)—the activities of the candidate effectors characterized in *N. benthamiana* cannot be directly extrapolated to tomato. Functional validation in tomato is therefore essential for accurately determining the roles of these elicitors and effectors during infestation. Such follow-up studies will provide definitive evidence of their contributions to host defense modulation and will guide the development of species-specific, targeted pest management strategies. Notably, the use of *N. benthamiana* as a screening platform in our work also carries practical implications. Given the documented susceptibility of tobacco to *T. absoluta*, our findings may offer early insights into potential molecular interactions relevant to protecting tobacco crops (Zhang et al., 2022). Overall, while *N. benthamiana* serves as a highly efficient preliminary screening system, species-specific confirmation in tomato remains indispensable for fully elucidating the biological functions of these insect-derived molecules.

In summary, we identified multiple candidate elicitors and effectors of the tomato leaf miner using multi-omics approach. Functional characterization in *N. benthamiana* validated three effectors—Ta21, Ta33, and Ta38—as key players, with their expression profiles further implicating potential roles in plant-feeding stages. Our findings highlight the dynamic interplay between *T. absoluta* secreted proteins and plant immunity, providing mechanistic insights into how insect herbivores circumvent host defense systems.

CRediT authorship contribution statement

Yumei Dong: Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Data curation. **Guolan Wu:** Methodology, Investigation. **Qiuyun Zhang:** Data curation. **Zhili Zhao:** Methodology. **Yunhua Zhang:** Methodology. **Qian Li:** Investigation. **Chuanlin Yin:** Writing – original draft, Funding acquisition, Data curation. **Pengjun Zhang:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2026.104500.

Data availability

Data will be made available on request.

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