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Harnessing plant-mediated RNAi for effective management of *Phthorimaea absoluta* by targeting *AChE1* and *SEC23* genes

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ABSTRACT

Tomato production on a global scale is under persistent pressure due to the devastating impact of *Phthorimaea absoluta* Meyrick (Lepidoptera: Gelechiidae), the South American tomato leaf miner. To combat this devastating pest, we explored the potential of plant-mediated RNA interference (RNAi) as a novel strategy for its management. Using transgenic techniques, we developed RNAi constructs (*p35S::dsAChE1, p35S::dsSEC23*) targeting crucial genes, *AChE1* and *SEC23*, in *P. absoluta*. These genes play pivotal roles in insect physiology and development. The transformation of tomato cultivar Rio Grande was carried out with these RNAi constructs using *Agrobacterium tumefaciens*. The results demonstrated a significant reduction in transcript levels of both *AChE1* and *SEC23* in *P. absoluta*. Silencing *AChE1* resulted in substantial mortality rates, reduced larval weight gain, and deformities, highlighting its pivotal role in insect survival. *SEC23* gene silencing also induced mortality and influenced insect physiology. Furthermore, we explored the susceptibility of *AChE1* to organophosphate insecticides, revealing its relevance in insecticide susceptibility. These findings support the potential of *AChE1* and *SEC23* as valuable targets for RNAi-based control of *P. absoluta* for the first time, providing multifaceted insights into insect physiology and insecticide susceptibility, thereby offering valuable insights for the development of effective strategies to mitigate the impact of this destructive pest.

Introduction

The South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae), stands as a significant threat to tomato production and the broader solanaceous crop family. Its accidental introduction into Spain in 2006 led to a rapid spread across Afro-Eurasia, warning global concerns (Giorgini et al., 2019; Gui-fen et al., 2020). This voracious pest displays a preference for attacking various solanaceous crops, including tomatoes, potatoes, peppers, eggplants, tobacco, and even weedy species like black nightshade. Once established, the larvae of *P. absoluta* tunnel into the leaves, fruits, and stems of tomato plants, consuming entire tissues when left uncontrolled (Desneux et al., 2010). With a significant ability for reproduction, *P. absoluta* can generate 10–12 generations annually, posing a severe threat to tomato crops (Nozad-Bonab et al., 2017). The larval phase includes four instars, with mature larvae typically pupating in the soil. Adult moths primarily exhibit crepuscular flight activity, remaining concealed within vegetation during the day, and displaying a preference for mating at the onset of the photo phase (Lee et al., 2014). The life cycle, spanning from egg to adult, extends over 26–38 days under field conditions, commonly resulting in overlapping generations (Guedes and Picanço, 2012).

The larval developmental stage of *P. absoluta* creates an extensive agricultural threat, causing significant economic losses in tomato

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cultivation (Rwomushana et al., 2019). The adverse effects of pest infestations on agricultural productivity demand significant attention, leading to multiple initiatives focused on pest management, primarily through the application of synthetic pesticides (Yadav et al., 2022; Chepchirchir et al., 2023). Synthetic insecticides have proven effective in controlling *P. absoluta* outbreaks, especially in newly invaded regions, primarily due to limited alternative options (Biondi et al., 2018). However, the widespread utilization of ininsecticides in pest management has raised significant concerns, including the emergence of insecticide resistance within *P. absoluta* populations (Campos et al., 2015; Roditakis et al., 2018). Additionally, the application of chemical pesticides can have adverse ecological consequences, potentially harming the natural enemies of the pest (Cloyd et al., 2012). The development of resistance to multiple insecticides within *P. absoluta* populations has emerged as a critical challenge in the management of this destructive tomato pest (Campos et al., 2017). Chlorantraniliprole, a widely employed insecticide, displayed early indications of resistance in Sicily, Italy, in 2012, subsequently resistant populations emerged in Brazil and the Eurasian region (Roditakis et al., 2015; Silva et al., 2016). Furthermore, Eurasian populations have exhibited resistance to indoxacarb, Spinosad, and emamectin benzoate (Roditakis et al., 2018). Given the ineffectiveness of chemical pesticides in controlling the infestation of the tomato leaf miner and the recurrent emergence of resistant populations, there is an urgent need to swiftly develop alternative methods for its management (Ciceoi et al., 2021). Biopesticides and natural enemies have been used to control this pest. Biopesticides, which are derived from natural sources such as neem oil and *Bacillus thuringiensis* (*Bt*), provide a targeted approach to controlling *P. absoluta* while minimizing damage to non-target organisms and reducing the usage of chemical pesticides (Mollá et al., 2011; Desneux et al., 2022). Additionally, *P. absoluta* populations have been suppressed by biological control mechanisms by natural enemies like the predator bug *Nesidiocoris tenuis* and the parasitoid wasp *Trichogramma pretiosum* (Faria et al., 2008; Calvo et al., 2012).

However, genetically modified (GM) plants present certain advantages over these conventional approaches in managing insect pests (Anderson et al., 2019). GM plants expressing insecticidal proteins have played a pivotal role in managing insect pests, becoming a widely accepted and extensive strategy for insect pest management in agriculture (James et al., 2015). Transgenic plants, exemplified by tomatoes engineered to produce *Bacillus thuringiensis* (*Bt*) toxins, have demonstrated resistance against lepidopteran insects, even though *P. absoluta* was not initially considered a primary target (Saker et al., 2011; Bergougnoux et al., 2014; Lu et al., 2012). However, the continuous expression of toxin proteins raises concerns about the development of insect resistance (Luttrell et al., 2004; Tabashnik et al., 2008). In response to these challenges, RNA interference (RNAi) technology has emerged as a promising alternative.

RNAi is a post-transcriptional gene silencing mechanism that regulates gene expression by interfering with protein production (Whyard et al., 2009; Son et al., 2017). The RNAi mechanism involves the use of double-stranded RNA (dsRNA) to silence specific target genes, making it a valuable tool for precise insect control (Tariq et al., 2023). Transgenic plants engineered to produce dsRNA targeting insect-pest genes have demonstrated their effectiveness in reducing pest damage by slowing insect growth and population growth, offering a precise and environmentally friendly approach to pest management (Mao et al., 2007; Zhu et al., 2012; Yao et al., 2013; Kamthan et al., 2015). Transgenic plants have emerged as a promising strategy for bolstering resistance against pest infestations through the expression of double-stranded RNA (dsRNA), with a focus on specific genes like *AChE1* and *SEC23*. *ACh*E1, or Acetylcholinesterase 1, plays a pivotal role in the nervous system of insects by catalyzing the conversion of acetylcholine into acetate and choline (Ye et al., 2017). This enzymatic activity is essential for proper nerve transmission. *AChE1* is highly expressed in insects and has been identified as a primary catalytic enzyme compared to *AChE*2, with point mutations in *AChE1* linked to pesticide resistance (Lee et al., 2007). Previous research has extensively documented the effects of *AChE* gene silencing on various aspects of insect biology, including survival, reproduction, embryo development, and growth (Revuelta et al., 2009; Kumar et al., 2009; Lu et al., 2012; Saini et al., 2018). To amplify the effectiveness of *AChE* gene silencing, the application of organophosphates (OPs) can be utilized synergistically. OPs function by inhibiting acetylcholinesterase (*AChE*), thereby preventing the breakdown of the neurotransmitter acetylcholine. This inhibition results in continuous nerve stimulation, ultimately causing paralysis and death in insects (Aroniadou-Anderjaska et al., 2023). When used in conjunction with transgenic plants that express dsRNA or dsRNA targeting *AChE*, OPs can significantly improve pest control outcomes. The dsRNA or dsRNA produced by the transgenic plants reduces *AChE* synthesis in the target pests, resulting in insufficient enzyme levels for normal neurological function. The application of OPs further inhibits these already reduced *AChE* levels, leading to a more comprehensive and effective disruption of the insect nervous system (Alex and Mukherjee, 2021; Neylon et al., 2022).

Similarly, *SEC23* genes play a crucial role in multiple biological processes, making them a promising RNA interference (RNAi) target for controlling *P. absoluta*. One example of their significance is evident in the study of *Drosophila melanogaster*, where mutations in *SEC23* and *SEC24CD*, known as haunted and ghost, impair cuticle development and disrupt cell polarity maintenance and the deposition of extracellular matrix/chitin (Norum et al., 2010). Given the essential role of *SEC23* in the production and preservation of the peritrophic membrane in the insect midgut, its silencing through oral ingestion of dsRNA can have a systemic impact on *P. absoluta* (Vélez et al., 2020). Moreover, *SEC23* mutations in *Drosophila* lead to the breakdown of septate junctions and epidermal adherents, similar to the effects observed in other RNAi studies (Hu et al., 2016; Li et al., 2018). *SEC23* is also integral to the *COPII* vesicle coat complex, facilitating rapid transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Barlowe et al., 1994; D'Arcangelo et al., 2013). Its role in vesicle cargo binding and membrane invagination via Sar1 activation underscores its importance as an RNAi target. Overall, *SEC23* emerges as a critical player in multiple cellular processes and a promising candidate for RNA interference (RNAi) strategies against *P. absoluta* (Norum et al., 2010; Hu et al., 2016; Li et al., 2018).

In this study, it was investigated that the plant-mediated RNAi can be a potential strategy to manage *P. absoluta*, a devastating pest. Using transgenic techniques, we targeted key genes in *P. absoluta*, including *AChE1* and *SEC23*. Hence, successful development of transgenic tomato plants expressing dsRNA highlights the promise of this approach for insect pest management and underscores the importance of understanding the roles of *AChE1* and *SEC23* in host-induced gene silencing.

Methodology

Insect rearing and host plants

Phthorimaea absoluta larvae were collected from Adana, Turkey, and brought to the controlled laboratory environment for further rearing. These conditions included maintaining a constant temperature of 25 ± 2 °C, maintaining a relative humidity range of 50 % to 60 %, light intensity of 4500 ± 500 lux, and a photoperiod of 14 h of light followed by 10 h of darkness.

The tomato cultivar 'Rio Grande' was utilized as the host plant for *P. absoluta* rearing. These tomato plants were cultivated in 2-liter disposable plastic pots filled with a mixture of perlite, peat moss, and vermiculite in a 1:1:1 ratio. The growth conditions provided for these tomato plants were carefully controlled to mirror the environmental parameters established for the rearing of the tomato leaf miner.

Molecular cloning and tomato transformation

The selection of target gene (*AChE1*) in this study was based on prior successful applications of RNAi for insect control (Kishk et al., 2017; Tian et al., 2022) retrieved from NCBI (KU985167.1) whereas *SEC23* gene was amplified from *P. absoluta* (uncharacterized earlier) using degenerate primers based on conserved regions found in homologous sequences, including those from *Helicoverpa armigera* (XM_021334926.1), *Spodoptera litura* (XM_02296176.1), *Plutella xylostella* (XM_011550139.1), *Bombyx Mori* (XM_004923303.3), and *Bombyx mandarina* (XM_028175529.1). Results successfully identified the *SEC23* gene in *P. absoluta*, utilizing M13- primers (Figure S1). To facilitate the production of double-stranded RNA (dsRNA) derived from the *AChE1* and *SEC23* target genes, the plasmid *pFGC5941*(Soliman et al., 2008) was chosen. Genes-specific primers (Table S1) were employed to amplify the target regions of *AChE1* and *SEC23*, with subsequent digestion by *Xho*1 and *Nco*1 and ligation into *pFGC5941* vector to create the *p35S::AChE1SENSE* and *p35S::SEC23SENSE* constructs. These constructs were then ligated with antisense fragments digested by *BamH*1 and *Xba*1, resulting in the formation of recombinant plasmids (*p35S::dsAChE1* and *p35S::dsSEC23*) (Soliman et al., 2008). The accuracy of both recombinant constructs was confirmed by vector specific PCR assays and restriction analysis.

For the subsequent transformation step, the *Agrobacterium* strain *EHA105* was used to transform the constructs (*p35S::dsAChE1, p35S::dsSEC23,* and *pFGC5941*) into Tomato Cultivar Rio Grande. The transformation procedure was followed as previously described by Hashmi et al. (2022). This resulted in the generation of transgenic Rio Grande plants expressing RNAi constructs.

Evaluation of RNAi lines

To confirm the presence of *p35S::dsAChE1, p35S::dsSEC23*, and the *pFGC5941*(empty vector) cassette in transgenic lines, PCR assays were conducted. Genomic DNA was extracted from the leaves of transformed tomato plants using the Thermoscientific Gene JET Plant Genomic DNA Purification Kit (Cat. No K0792). Specific primers for PCR detection in ds*AChE1* and ds*SEC23* transgenic plants, as detailed in Table S1, were used.

From the pool of PCR-positive primary transformants, five randomly selected transgenic plants from both ds*AChE1* and ds*SEC23* groups were subjected to qRT-PCR analysis to assess *AChE1* and *SEC23* transcript levels. Total RNA extraction was conducted from the leaves of both transgenic and non-transgenic plants. For RNA extraction, the AurumTM total RNA Mini Kit (Bio-Rad, Cat #732–6820) was employed, and RNA concentration was determined following the procedure outlined by Dangol et al. (2020). Then, first-strand cDNA synthesis was carried out using the Revert Aid cDNA Synthesis Kit (Thermo Scientific #K1621). Finally, the expression levels of ds*AChE1* and ds*SEC23* constructs in the selected transgenic plants were quantified using qRT-PCR, following the procedure as described by Hussain et al. (2019). The expression levels of ds*AChE1* and ds*SEC23* constructs were calculated relative to the control plants.

Bioassays for evaluating the transgenic plant effects on P. absoluta

To evaluate the impact of ds*AChE1* and ds*SEC23* transgenic plants on *P. absoluta*, a larvae-feeding bioassay was conducted. Leaves from ds*AChE1*, ds*SEC23* transgenic plants, and control non-transgenic plants were detached for this purpose. Second, third, and fourth instar larvae were placed on these leaves within individual petri dishes (90 mm) containing transgenic leaves separated from their stems, with petioles wrapped in moistened cotton (Fig. 2). The larvae were fed until pupation, with daily plant foliage replacement. Controls received non-transgenic leaves and empty vectors (*pFGC5941*). Assessments of insect mortality rates were conducted on days 2, 3, 4, and 6. Each dsRNA feeding bioassay included three replications with 30 larvae per group (ds*AChE1* and ds*SEC23*). The larvae were continuously fed with the selected foliage. Further observations included surviving larvae weight analysis, and pupae to adult conversion according to established criteria (Naqqash et al., 2020; Hussain et al., 2019).

To assess the effect of transgenic plants on the fecundity of adult insects. Adult *P. absoluta* emerged from pupae were paired and placed on tomato plants, and the number of eggs laid per female was recorded during the initial 10 days post-emergence. Additionally, the effects of plant-expressing ds*AChE1* and ds*SEC23* on transcript levels of the target genes in larvae were quantified via qRT-PCR using specific primers (Table S1), with ribosomal protein 18 (RPS18) serving as the internal control gene following Yang et al. (2015) method. Total RNA was extracted from larvae three days after the feeding bioassay using the AurumTM Total RNA Mini Kit (Bio-Rad, Cat #732–6820). Data analysis was carried out using a Real-Time PCR Detection System (Qiagen, Netherlands), and relative gene expression was calculated using the Livak and Schmittgen (2001) equation.

Synergism of dsRNA-AChE1 with organophosphate

For this experiment, second instar larvae were chosen as they are widely preferred by researchers for experimental purposes (Zhu et al., 2011). To evaluate the effectiveness of Hypnose Total, a commercial insecticide containing 500 g/L Primiphos Methyl and 7.5 g/L Emamectin Benzoate, against *P. absoluta*, transgenic plants producing ds*AChE1* were irrigated with various concentrations of the standard insecticide (1 %, 5 %, 10 %, 20 %, 40 %, and 80 %). Hypnose Total was generously provided by Safa Tarim Insecticide (https://www.safatarim.com/) upon special request for the experiment's specific purpose. The remaining procedures and larval incubation followed the methodology described previously.

Fecundity evaluation of adults fed with plant expressing dsSEC23 extract

For this experiment, leaves and flower extracts from ds*SEC23* expressing plants were utilized to prepare a solution using a honey and water composition. Five pairs of newly emerged *P. absoluta* adults, obtained from pupae, were employed. The treatment groups were fed with the solution of transgenic plants, while the control group received non-transgenic plant solution. Subsequently, the insects were allowed to lay eggs on fresh tomato plants. This experimental setup was replicated three times. The key factors assessed in this experiment included the total number of eggs produced in each treatment group and the number of viable eggs. Viable eggs were determined as those from which larvae successfully emerged, while non-viable eggs were counted as those that failed to hatch.

Data analysis

The bioassay data were analyzed using standard statistical techniques. The mortality data were subjected to arcsine transformation prior to undergoing ANOVA analysis at a significance level of 5 %. Tukey's multiple comparison test was employed to assess the differences of treatments from each other ($P \leq 0.05$). The results of the qRT-PCR analysis were assessed using the paired *t*-test. All data analyses were conducted using Statistix 8.1 (Analytical Software, 2005) to ensure robust statistical evaluation.

Results

Development of expression cassettes

To advance the development of RNA interference (RNAi) constructs designed for precise gene silencing, we engineered *p35S::dsAChE1* and *p35S::dsSEC23* constructs (Figure S2 A-B). These carefully designed

constructs were strategically introduced into tomato plants through a very efficient *Agrobacterium*-mediated transformation system. Our search for optimized transformation efficiency led us to employ 14-day-old tomato explants, subjecting them to *Agrobacterium* suspension for three distinct constructs: *p35S::dsAChE1, pFGC594::dsSEC23* and *pFGC5941*. The significantly higher transformation efficiency, rising to 27.37 %, was observed when explant tissues were exposed to bacterium cells for 15 min (Table S2).

Successful transformation mechanisms depend on the presence of appropriate antibiotic concentrations, serving as selection markers, within the regeneration media. In our experiments, we used *Phosphinothricin* (*PPT*) as the selection agent. The evaluation of different *PPT* concentrations (ranging from 0.1 to 0.5 mg/L) during the initial selection phase revealed the highest transformation efficiency of an impressive 40.82 %, with 0.5 mg/L of *PPT*. Subsequent attempts with varying *PPT* concentrations resulted in a gradual decline in regeneration and transformation efficiency, with reduced selective pressure observed at 0.1 mg/L. Conversely, maximum regeneration efficiency was achieved at 1.5 mg/L of 6-Benzylaminopurine (BAP), confirming with findings reported by Hashmi et al. (2022).

In the shoot elongation phase, we used combinations of Gibberellic acid (GA3) and BAP at different concentrations. This resulted in the highest shoot induction frequency, with a significant proportion of shoots originating from a single callus, subsequently categorized as different shooting events (Figure S3). Following the acclimatization of 100 plants, 20 putative transgenic Rio Grande plants (9 *p35S::dsAChE1*, 8 *p35S::dsSEC23*, 3 *pFGC5941*) were closely monitored for growth and nutrient requirements.

Evaluation of primary transformants

Putative transgenic plants were generated through *Agrobacterium*-mediated transformation methods, yielding 20 independent positive transgenic plants as confirmed by PCR analysis (Figure S4 a,b). These PCR-positive plants were tested to see if they expressed ds*AChE1* and ds*SEC23*, using qPCR. The results from the qRT-PCR analysis clearly showed that transgenic plants expressed ds*AChE1* and ds*SEC23*, while the control plants did not. Additionally, there were noticeable differences in the levels of ds*AChE1* and ds*SEC23* expression among the transgenic plants (Fig. 1a,b). These findings revealed through the qRT-PCR analysis, highlighted the significant variation in expression levels between ds*AChE1* and ds*SEC23* in the transgenic plants. For instance, ds*AChE1* showed a substantial 55.5-fold increase in expression (Fig. 1a), while *SEC23* dsRNA displayed a 45-fold increase (Fig. 1b). Consequently, we selected transgenic plants with higher dsRNA expression levels, which exhibited similar appearances to regular plants, for further

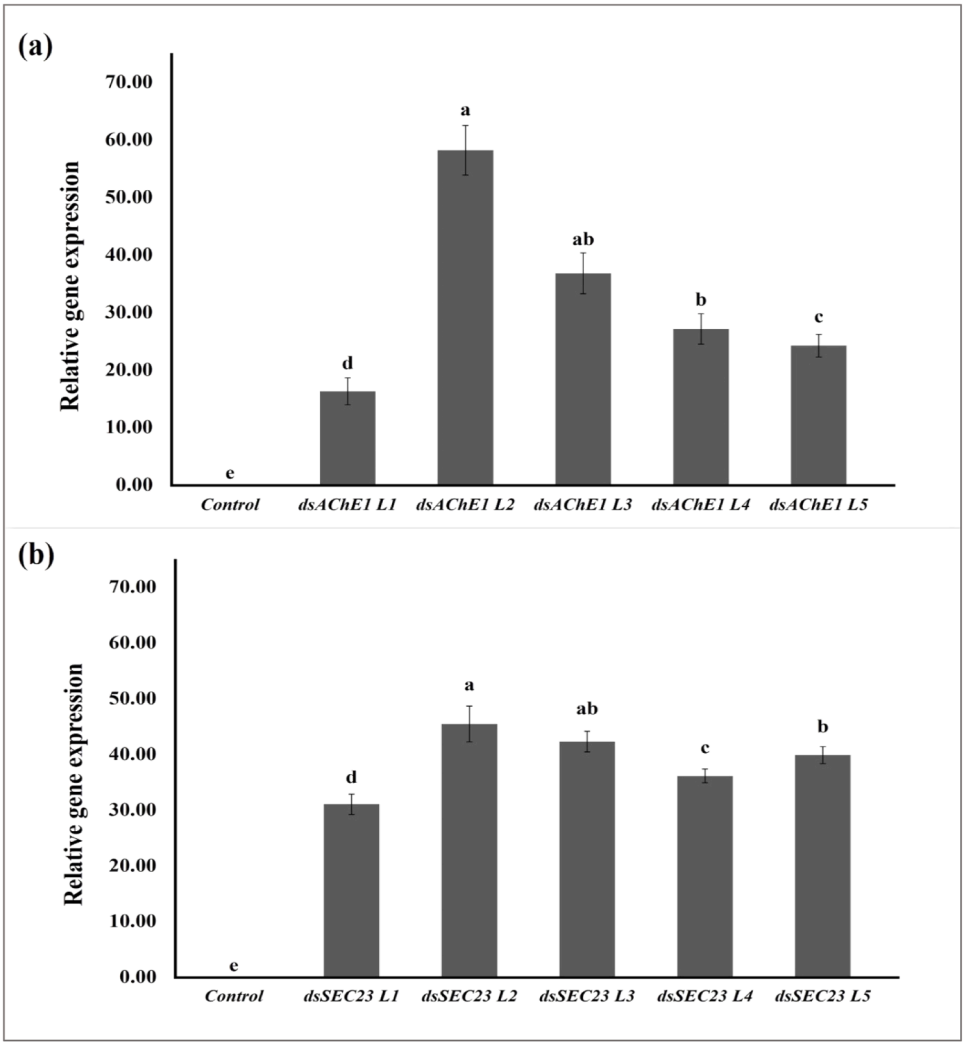

Fig. 1. mRNA expression for *AChE1* and *SEC23* detected in Rio Grande primary transformants (a) *AChE1* transcript level in T0 tomato lines compared to control non-transgenic plants (b) Shows T_0 tomato lines transcript level of *SEC23* compared to control. Mean values with standard error (SE) represented in columns, significance differences indicated by distinct letters and determined via ANOVA followed by Tukey's HSD test at a 5 % significance level.

assessments of RNAi efficiency and insect resistance.

Insect bioassays

The bioassays were conducted in controlled laboratory settings to ensure precision and consistency in the data collection process. We meticulously designed and executed these experiments to gain insights into the potential utility of dsRNA/dsRNA-expressing transgenic plants as a novel approach to pest management (Fig. 2). The objective was to assess the impact of transgenic plants on various aspects of *P. absoluta* biology, including mortality rates, mRNA levels of the targeted genes, weight gain, and fecundity.

Mortality rates assessment

The investigation aimed to assess the efficacy of dsRNA treatments targeting *AChE1* and *SEC23* genes in various developmental stages of *P. absoluta* larvae. The RNA interference (RNAi) approach directed at both *AChE1* and *SEC23* genes resulted in high mortality rates across various developmental stages. Statistical analysis revealed significant differences in mortality rates between the ds*AChE1* and ds*SEC23* treatment groups compared to the non-transgenic and empty groups, with a significance level of $P \leq 0.05$. Specifically, 2nd instar larvae exhibited the most substantial impact, with a remarkable mortality rate of 14.46 % ± 0.25 % and 18.18 ± 0.25 following a 4-day exposure to ds*AChE1* and ds*SEC23*. In addition, 3rd instar larvae showed significant susceptibility to the RNAi treatment, recording a mortality rate of 11.11±0 and 14.46 ±0.25 upon exposure to leaves expressing ds*AChE1* and ds*SEC23* (Table 1). Mortality assessment for 4th instar larvae was excluded from the assessment due to pupation initiation three days into the feeding bioassay. Conversely, control groups fed non-transgenic leaves or empty vector-transformed tomato leaves exhibited no mortality. These findings underscore the potential utility of ds*AChE1* and ds*SEC23* in effectively inducing mortality in *P. absoluta* 2nd and 3rd instar larvae following a 4-day exposure period, highlighting their value as essential tools in integrated pest management strategies.

Table 1
The mortality rates (Mean ± SEM) of *P. absoluta* 2nd and 3rd instar larvae following a 4-day exposure to various treatments.

Treatments	% Mortality (Mean±SEM)	
	2nd instar larvae 4 DAT	**3rd instar larvae** 4 DAT
Non-Tran	0 ± 0 b	0 ± 0 b
Empty	0 ± 0 b	0 ± 0 b
dsAChE1	14.46 ± 0.25 a	11.11 ± 0 a
dsSEC23	18.18 ± 0.25 a	14.46 ± 0.25 a

SEM = Standard Error Mean, mean values followed by the different letter in the same column are statistically different ($P \leq 0.05$), DAT= Days after treatment.

Assessing transcript levels of AChE1 and SEC23 via qRT-PCR

To evaluate the efficacy of plant-mediated RNAi in *P. absoluta*, we conducted real-time PCR analysis to assess the transcript levels of *AChE1* and *SEC23* genes in different larval instars. In the 2nd instar larvae, the expression of ds*AChE1* and ds*SEC23* had a significant impact on the relative gene expression of the targeted genes when compared to the control group. Larvae fed on leaves expressing ds*AChE1* and ds*SEC23* exhibited significantly lower expression levels of *AChE1* (0.46721) and *SEC23* (0.47180), respectively, in contrast to the control (Fig. 3a) ($P \leq 0.05$). These results indicate a knockdown efficiency of approximately 40 to 60 % using plant-mediated RNAi for *P. absoluta* in the 2nd instar larvae.

In the 3rd instar larvae, a similar trend was observed, with a significant reduction in the gene expression of both *AChE1* and *SEC23* compared to the control group. The relative expression level of *AChE1* was notably lower (0.48124) in 3rd instar larvae that had been fed dsRNA-expressing leaves for 96 h, while no silencing effect was observed

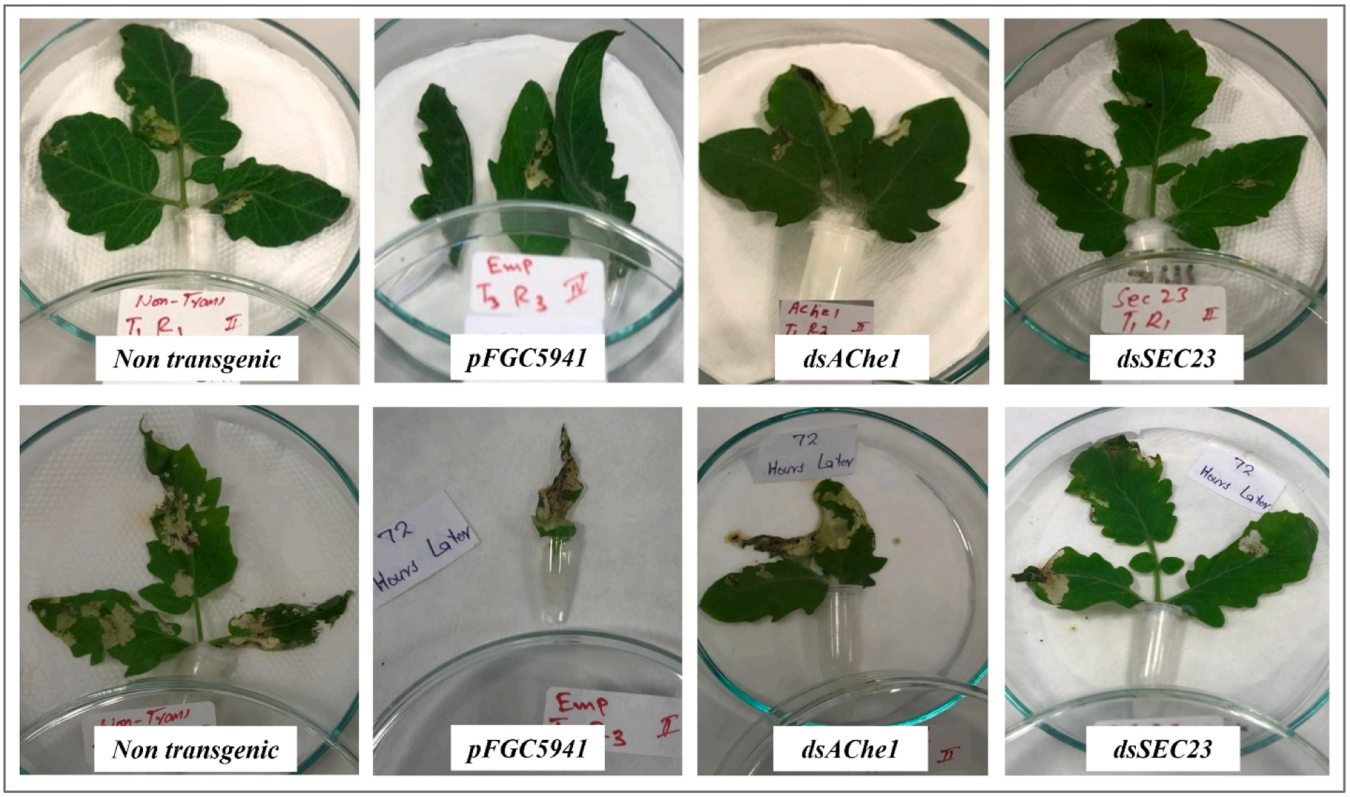

Fig. 2. Comparative images showcasing the feeding of *Phthorimaea absoluta* and the varying extent of damage in transgenic and control lines.

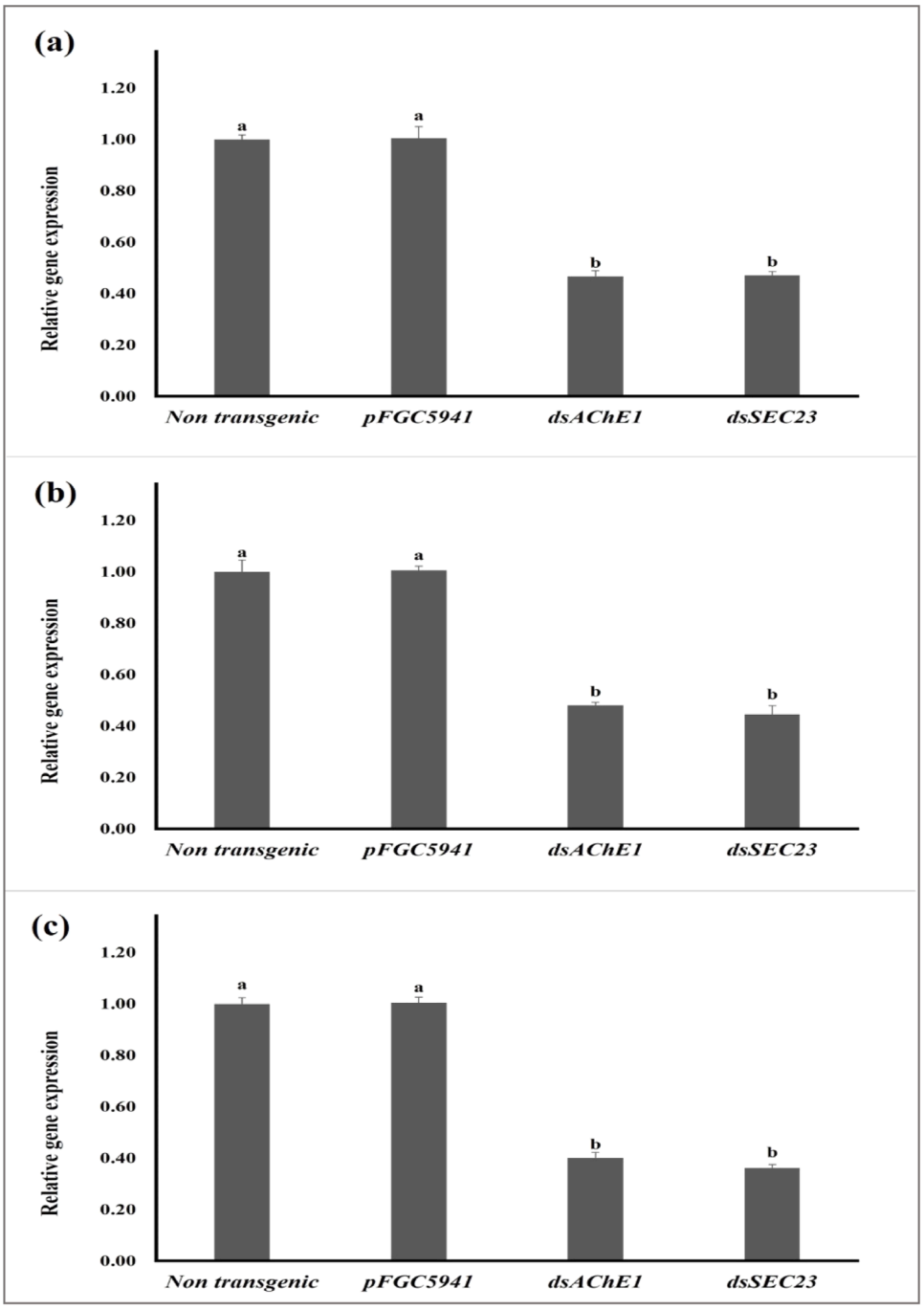

Fig. 3. qRT-PCR analysis in *P. absoluta* larvae after feeding on dsRNA expressing leaves (a) Transcript level of *AChE1* and *SEC23* in 2nd instar *P. absoluta* larvae fed on non-transgenic, empty *pFGC5941* plasmid, ds*AChE1* and ds*SEC23* expressing plants (b) Transcript level of *AChE1* and *SEC23* in 3rd instar *P. absoluta* larvae fed on non-transgenic, empty *pFGC5941* plasmid, ds*AChE1* and ds*SEC23* expressing plants (c) Transcript level of *AChE1* and *SEC23* in 4th instar *P. absoluta* larvae fed on non-transgenic, empty *pFGC5941* plasmid, ds*AChE1* and ds*SEC23* expressing plants. Mean values with standard error (SE) represented in columns, significance differences indicated by distinct letters and determined via ANOVA followed by Tukey's HSD test at a 5 % significance level.

in the control group. Likewise, larvae fed ds*SEC23* exhibited reduced relative expression of *SEC23* (0.44541) in the 3rd instar compared to the control (Fig. 3b) ($P \leq 0.05$).

Moving to the 4th instar larvae, we continued to investigate the relative expression of the targeted genes *AChE1* and *SEC23*. The control expression was normalized to 1.0000 for relative expression analysis. Clearly different expression patterns were observed between the treatment and control groups. Significant down-regulation of *AChE1* (0.4014) and *SEC23* (0.3624) expression levels was recorded in the 4th instar larvae subjected to dsRNA treatment compared to the control group (Fig. 3c). By this stage, it is evident that the targeted genes had undergone significant silencing, with an estimated knockdown efficiency of approximately 60 %. These findings highlight the effectiveness of plant-mediated RNAi in modulating the transcript levels of *AChE1* and *SEC23* in *P. absoluta* larvae across different instars

Weight gain analysis

The impact of dsRNA ingestion on the weight gain of different *P. absoluta* larval instars was investigated. The larvae across various

instars that consumed dsRNA (*AChE1* and *SEC23*) exhibited statistically significant reduced body weights compared to those that fed on non-transgenic and empty vector-transformed leaves ($P \leq 0.05$). The larvae fed with non-transgenic plants exhibited the highest weight gain across all instar stages, with mean values of approximately 0.006 mg, 0.005 mg, and 0.005 mg for the 2nd, 3rd, and 4th instar larvae, respectively. Similarly, the larvae fed with plants carrying empty *pFGC5941* demonstrated substantial weight gain, albeit slightly lower than the non-transgenic group. In contrast, the ds*AChE1* and ds*SEC23* treatments resulted in significantly reduced weight gain, indicating a pronounced inhibitory effect on larval growth. The larvae fed with plants carrying ds*AChE1* showed mean weight gains of approximately 0.003 mg, 0.002 mg, and 0.002 mg for the 2nd, 3rd, and 4th instar larvae, respectively, while the larvae fed with plants carrying ds*SEC23* exhibited the lowest weight gain, with values around 0.002 mg, 0.001 mg, and 0.001 mg for the respective instar stages. Differences in the body shape of some 2nd instar 3rd instar larvae are highlighted in (Fig. 5a,b), reflecting the impact of dsRNA treatments. These findings underscore the significant silencing impact of *AChE1* and *SEC23,* which significantly impair the growth of larvae compared to the control groups.

Adult emergence rate from pupae

Following the ingestion of transgenic tomato leaflets expressing dsRNA by *P. absoluta* larvae, we closely monitored their developmental stages and assessed their survival rate, particularly focusing on the emergence of adult individuals. Notably, the pupae that had developed from the control group displayed normal health and larger sizes in comparison to those that had been fed transgenic leaves. The emergence of adult individuals was carefully recorded after subjecting larvae to dsRNA treatments, revealing statistically significant differences. Among the larval stages that ingested dsRNA-*SEC23*, the 4th exhibited the lowest rate of adult emergence, with only 25 % successfully transitioning into adults. In comparison, dsRNA-*AChE1* treatment yielded a relatively higher emergence rate of 46 %. Meanwhile, the control group demonstrated a robust emergence rate of 90 % (Fig. 6). Cumulative mortality rates in dsRNA-treated individuals reached approximately 50 % to 70 %, which was significantly higher than that observed in the control group (Figs. 6). Furthermore, it was evident that adults emerging from pupae that had originated from larvae fed on dsAChE1 and ds*SEC23* exhibited varying degrees of abnormality in their appearance (Fig. 7). This observation underscores the impact of dsRNA treatments on the development and emergence of adult individuals.

Fecundity evaluation of the emerged adults

The experiment aimed to evaluate the fecundity of the emerged adults across different treatments and instar stages. The hatching rate was calculated as the percentage of hatched eggs relative to the total number of eggs laid. This metric allowed for a comprehensive comparison of fecundity across different treatments and developmental stages. The non-transgenic group exhibited the highest fecundity across all stages, with the 2nd instar showing a hatching rate of 90.65 %, the 3rd instar 94.12 %, and the 4th instar 88.81 %. The empty vector group maintained relatively high fecundity, second only to the non-trans group. Conversely, the ds*AChE1* treatment group consistently displayed the lowest fecundity, with hatching rates of 50.00 %, 58.82 %, and 57.14 % for the 2nd, 3rd, and 4th instar larvae, respectively. The ds*SEC23* group also showed reduced fecundity, particularly notable in the 3rd instar larvae with a hatching rate of 61.29 %. These findings suggest that the gene-silencing treatments ds*AChE1* and ds*SEC23* adversely affect the reproductive capacity of the emerged adults, with the ds*AChE1* treatment showing the most pronounced reduction in fecundity. These results provide valuable insights into the impact of gene-silencing treatments on the reproductive biology of the *P. absoluta*, highlighting potential applications and considerations for pest management strategies.

Fecundity evaluation of adults fed with plant expressing dsSEC23 extract

To evaluate the actual impact of ds*SEC23* on the fecundity and egg viability of *P. absoluta* adult insects, an experiment involving the ingestion of dsRNA plant leaf extract was conducted. The proportion of egg-laying by female insects exposed to dsRNA plant leaf extract was determined, and the viability of the resulting eggs was assessed. The results revealed a significant reduction in the fecundity of *P. absoluta* adult insects due to RNAi, particularly when targeting ds*SEC23*. *P.*

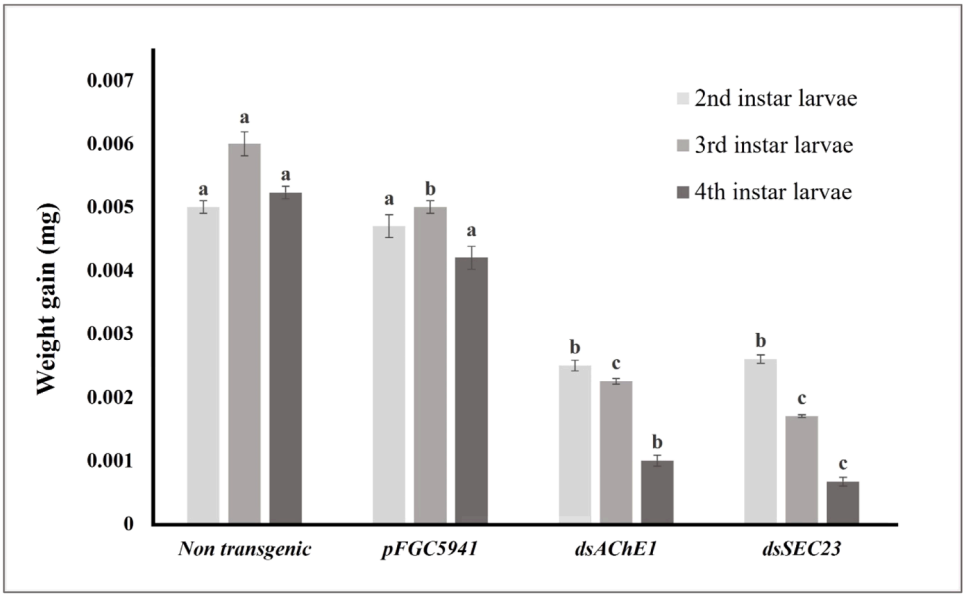

Fig. 4. Weight gain (mg) in 2nd 3rd and 4th instar *P. absoluta* larvae fed on non-transgenic, empty *pFGC5941* plasmid, ds*AChE1* and ds*SEC23* expressing plants. Mean values with standard error (SE) represented in columns, significance differences indicated by distinct letters and determined via ANOVA followed by Tukey's HSD test at a 5 % significance level.

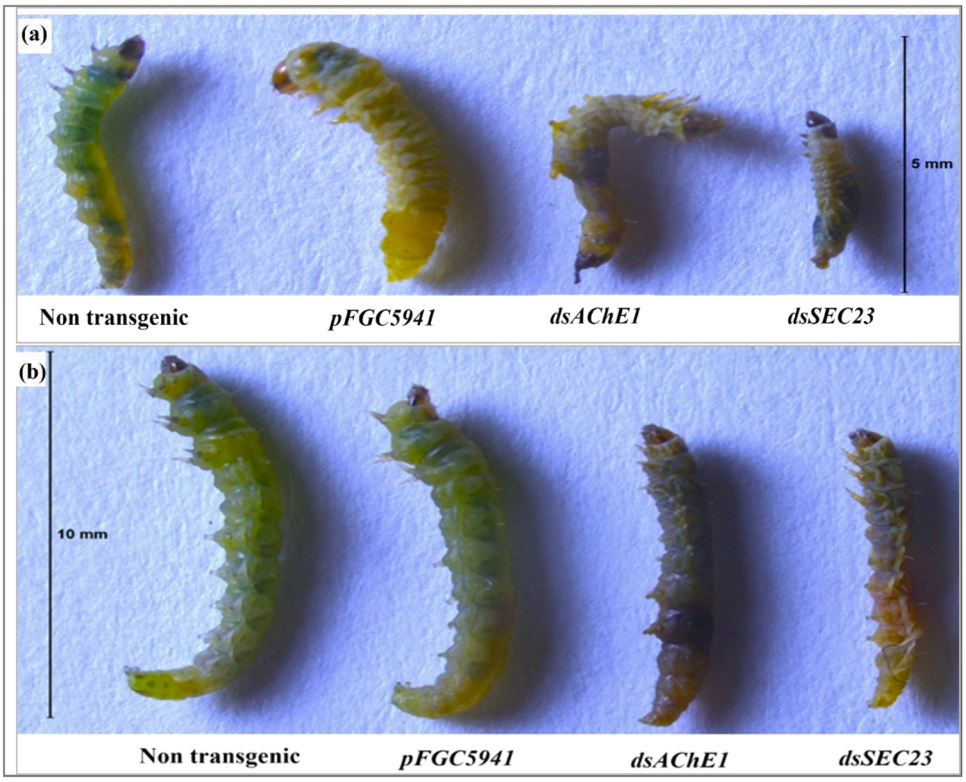

Fig. 5. Comparative images showcasing the morphological changes in *Phthorimaea absoluta* larvae fed on non-transgenic, empty *pFGC5941* plasmid, ds*AChE1* and ds*SEC23* expressing plants.

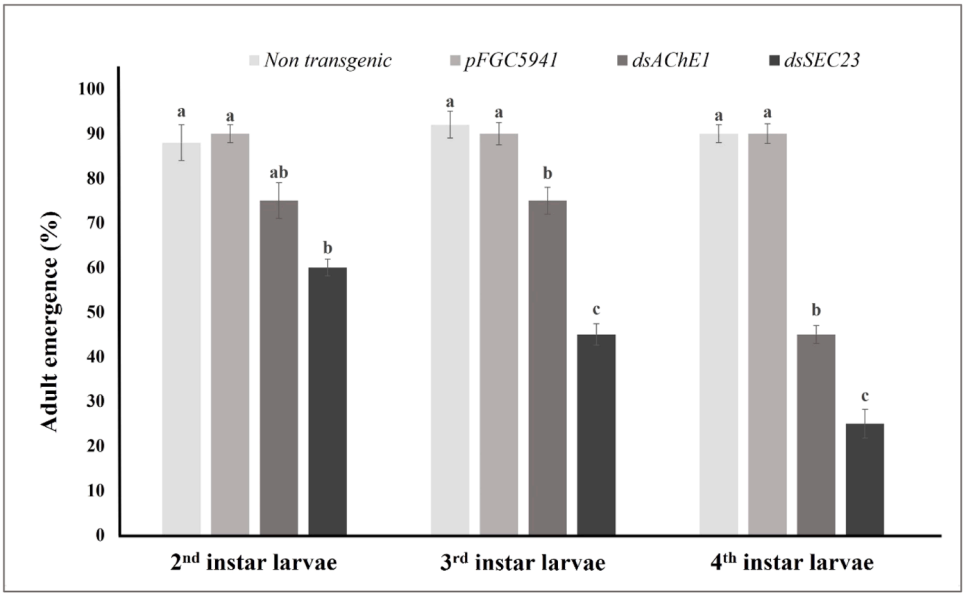

Fig. 6. RNAi effects *P. absoluta* adult's emergence from 2nd,3rd and 4th instar larvae fed on non-transgenic, empty *pFGC5941* plasmid, ds*AChE1* and ds*SEC23* expressing plants.. Mean values with standard error (SE) represented in columns, significance differences indicated by distinct letters and determined via ANOVA followed by Tukey's HSD test at a 5 % significance level.

absoluta adult pairs that ingested ds*SEC23* exhibited reduced egg viability compared to those fed on non-transgenic leaf extract (Table 2). In the control (non-transgenic) group, the mean number of eggs laid was 181.33 (± 5.29 SE), with a mean of 161.60 (± 4.69 SE) eggs hatched, resulting in a hatching rate of approximately 89.11 % (Table 2). Conversely, the ds*SEC23* treatment group exhibited a mean number of eggs laid of 138.93 (± 4.99 SE) and a mean of 95.40 (± 8.84 SE) eggs hatched, corresponding to a significantly lower hatching rate of approximately 68.65 % (Table 3). Targeting ds*SEC23* genes resulted in a significant reduction in both egg-laying and the hatching rate of eggs and thus ensured the significant impact of ds*SEC23* on the fecundity and egg viability of *P. absoluta* Table 3)(.

Fig. 7. Comparative images showcasing the deformities in insects fed on ds*AChE1* and ds*SEC23* expressing plants compared to non-transgenic (control) plants.

Table 2
Comparison of the mean number of eggs laid by *Phthorimaea absoluta* Adults and the mean number of hatched eggs relative to control, and their percent hatching rate, illustrating the impact of RNAi on the fecundity of emerged adults.

Treatment	2nd instar larvae			3rd instar larvae			4th instar larvae		
	Number of eggs (means ± SE*)	Number of hatched eggs (means ± SE)	Hatching rate (%)	Number of eggs (means ± SE*)	Number of hatched eggs (means ± SE)	Hatching rate (%)	Number of eggs (means ± SE*)	Number of hatched eggs (means ± SE)	Hatching rate (%)
Non-Trans	20.00 ± 2.14	18.13 ± 0.98	90.65	21.25 ± 3.28	20.00 ± 3.76	94.12	21.89 ± 2.38	19.44 ± 0.63	88.81
Empty	17.50 ± 1.67	15.00 ± 1.56	85.71	18.33 ± 1.88	17.22 ± 2.74	93.94	20.89 ± 2.13	17.79 ± 1.49	85.17
dsAChE1	16.00 ± 1.89	8.00 ± 0.24	50.00	17.00 ± 1.43	10.00 ± 0.75	58.82	15.40 ± 1.73	8.80 ± 0.63	57.14
dsSEC23	18.50 ± 2.09	12.50 ± 1.07	67.57	15.50 ± 1.56	9.50 ± 0.53	61.29	19.00 ± 2.52	11.50 ± 1.41	60.53

*Standard error.

Table 3
Comparative analysis of oviposition and percent hatching rates in *Phthorimaea absoluta* adults following consumption of plant juice from ds*SEC23*-expressing plants versus non-transgenic plants.

Treatment	Number of eggs (means ± SE*)	Number of hatched eggs (means ± SE)	Hatching rate (%)
Control (non-transgenic)	181.33 ± 5.29	161.60 ± 4.69	89.11 %
dsSEC23	138.93 ± 4.99	95.40 ± 8.84	68.65 %

*Standard error.

Synergism of dsRNA-AChE1 with organophosphate

In our investigation, we explored the potential synergistic effects of dsRNA-*AChE1* in combination with Hypnose total, an organophosphate insecticide, on the 2nd instar larval stage of *P. absoluta.* Statistically significant differences among treatments were observed ($F = 125.02$, df=6,14, $P < 0.05$) (Fig. 4). Notably, when compared to larvae fed on the control diet (non-transgenic), those consuming dsRNA-*AChE1*-expressing plants irrigated with Hypnose total exhibited significantly elevated mortality rates. The highest mortality rates were recorded for the ds*AChE1* treatment in combination with 80 % of the recommended insecticide dose, where all tested insects died after 96 h of treatment (Fig. 4). Afterward, the ds*AChE1* treatment combined with a 40 % recommended dose resulted in an 80 % mortality rate. Even when the insecticide dose was reduced, it continued to induce significantly higher mortality compared to the control group, except for the ds*AChE1* treatment combined with a 1 % recommended dose.

The assessment of larval body weight following exposure to different doses of ds*AChE1* in combination with organophosphate insecticide provides valuable insights into the insects performance and damage potential. While no significant difference was observed among treatments ($F = 1.58$, df=6182, $P = 0.15$), notable variations in larval weight gain emerged when considering specific treatment combinations. Within the tested treatments, ds*AChE1* treatments coupled with 40 % and 80 % of the recommended insecticide dose resulted in reduced weight gain. Notably, the most significant weight gain was recorded in the ds*AChE1* treatment combined with a 5 % recommended dose, where larvae exhibited an increase in weight of 0.0061 mg after a 3-day exposure period (Table 4).

Discussion

Insect pests have traditionally been managed using chemical control methods. However, the development of resistance and environmental safety concerns have driven scientists to seek better alternatives (Roditakis et al., 2018). *Phthorimaea absoluta*, a major pest of tomato plants, is currently controlled using chemical insecticides, but this approach has led to complications such as resistance development and

Table 4
Mortality and weight gain of 2nd instar of *P. absoluta* fed with ds*AChE1* expressing potato plants treated with various doses of primiphos methyl+ emamectin benzoate.

Treatment	% Mortality (Mean ±SEM)	Larval weight gain (mg) (Mean±SEM)
ds*AChE1* + No Chemical	13.51 ± 0.76 a	0.0032 ± 0.0009
ds*AChE1*+ 1 % recommended dose	17.15 ± 0.31 ab	0.0031 ± 0.0007
ds*AChE1*+ 5 % recommended dose	28.06 ± 1.12 b	0.0061 ± 0.0154
ds*AChE1*+ 10 % recommended dose	45.67 ± 0.19 c	0.0026 ± 0.0011
ds*AChE1*+ 20 % recommended dose	56.82 ± 0.33 c	0.0027 ± 0.0008
ds*AChE1*+ 40 % recommended dose	80.37 ± 0.31 d	0.0020 ± 0.0008
ds*AChE1*+ 80 % recommended dose	100.00 ± 0.0 e	0.0021 ± 0.0010

Different letters within the column indicate statistically significant variations between the treatments ($P \leq 0.05$).

negative impacts on non-target species (Guedes and Siqueira, 2012; Barros et al., 2015). To address these issues, researchers have developed RNAi technology, which utilizes gene silencing to control pests (Lindbo and Dougherty 2005; Tariq et al., 2023). This technology has shown significant results across various insect orders including Hemiptera (Zha et al., 2011; Bansal and Michel, 2013), Diptera (Puglise et al. 2016), Coleoptera (Prentice et al., 2015; Laudani et al., 2017), Hymenoptera (Hunter et al., 2010; Meng et al., 2020), Orthoptera (Hoang et al., 2022; Rana et al., 2023), Isoptera (Z Mogilicherla et al., 2023; Suzuki et al., 2023) and Lepidoptera (Kebede and Fite, 2022; Kottaipalayam-Somasundaram et al., 2022). Currently, RNAi is applied through artificial feeding and injection methods, which, although effective in controlled environments, are impractical for large-scale agricultural use (Yan et al., 2020; He et al., 2022). In an effort to find more practical solutions, Majidiani et al. (2019) investigated the effect of silencing acetylcholinesterase (*AChE*), ryanodine (*RyRs*), and nicotinic acetylcholine alpha 6 (*nAChRs*) receptors. Their study on control strategies against *P. absoluta* demonstrated the potential of using both injection and root administration methods for effective RNAi. However, there is a need to develop transgenic plants that can express RNAi constructs, providing a sustainable and effective solution for managing *P. absoluta* and other insect pests in the field (Camargo et al., 2017; Pizetta et al., 2022). Such advancements could revolutionize pest management by offering targeted control with reduced environmental impact. In the creation of transgenic plants that express non-endogenous dsRNA, forming either a long hairpin (dsRNA) or short interfering RNA (siRNA), researchers commonly employ the *Agrobacterium tumefaciens* mediated transformation method (Guo et al., 2015; Mamta et al., 2016; Kumar et al., 2012). This technique has been instrumental in advancing genetic modification research. The process of identifying the appropriate target gene for RNAi can be a challenging endeavor, as it holds significant importance in determining the successful results of the RNAi experiment in transgenic plants (Yu et al., 2014). Herein, we employed the *AChE1* and *SEC23* genes as key components in the development of transgenic crops, drawing from recent research and genetic engineering advancements.

In this study, the targeted silencing of both the *AChE1* and *SEC23* genes through RNAi has yielded significant insights into their pivotal roles in *P. absoluta* and their potential as promising targets for pest management strategies. Furthermore, it had a pronounced impact on larval weight gain, adult emergence, and fecundity rates. The downregulation of *AChE1* transcript levels further confirmed the effectiveness of this RNAi approach. Silencing of the *AChE1* gene resulted in substantial mortality rates, indicating its critical role in insect survival. Likewise, Saini et al. (2018) reported that the transgenic-treated *H. armigera* displayed a larval mortality rate of 25 %, adult deformity at 20 %, and a substantial 70–80 % reduction in acetylcholinesterase mRNA levels in defective adults. Also, the silencing of *AChE1* in *Chilo suppressalis* resulted in up to 25 % mortality and 50–70 % in transcript levels compared to control (Hui et al., 2011). *Acetylcholinesterase,* as revealed in studies by Lu et al. (2012) on *Tribolium castaneum*, Zhao et al. (2019) on Blattella germanica, Kola et al. (2019) on *Scirpophaga incertulas*, He et al. (2012) on *Plutella xylostella*, Faisal et al. (2021) on *Myzus persicae*, and Wang et al. (2014) on *Plutella xylostella*, has been identified as a pivotal target in RNAi investigations, resulting in multifaceted effects such as increased mortality, reduced weight, diminished transcript levels, deformities, and heightened sensitivity to insecticides (Revuelta et al., 2009). Additionally, we aimed to explore the role of the *AChE1* gene in neurotransmission and its vulnerability as a target for anticholinesterase insecticides. To investigate, we applied the organophosphate insecticide Hypnose total (500 g/L Primiphos Methyl + 7.5 g/L Emamectin Benzoate), resulting in a notable increase in mortality rates among the targeted insects. The reported study is supported by Lu et al. (2012) pioneering work, which combined an RNAi bioassay with insecticide application, revealing *AChE1* pivotal role in cholinergic functions and its susceptibility as a target for anticholinesterase insecticides. Similarly, the silencing of the *SEC23* gene also induced mortality, suggesting its importance in insect viability. Additionally, it influenced larval weight gain, adult emergence, and fecundity rates. The reduction in *SEC23* transcript levels confirmed the success of this RNAi intervention. In line with our research, Cedden et al. (2023) conducted feeding bioassays on *P. chrysocephala* adults, demonstrating that ds*SEC23* resulted in a mortality rate of 76 %. The significance of the *SEC23* gene as a vital target for RNAi-mediated pest control has been supported by several noteworthy studies in insects. Zhu et al. (2011) conducted research on *Leptinotarsa decemlineata*, while Guo et al. (2023) focused on *Henosepilachna vigintioctopunctata*. Additionally, Tian et al. (2022) investigated *Colaphellus bowringi*, Zhang et al. (2023) explored *Hyphantria cunea*, and Koo et al. (2020) delved into *Lasioderma serricorne*. Across these diverse pest species, the outcomes were consistently compelling, with the silencing of the *SEC23* gene effectively inducing mortality and deformities in the targeted pests.

In conclusion, this study has revealed the pivotal roles of *AChE1* and *SEC23* genes in *P. absoluta,* affirming their potential as critical targets for innovative pest management approaches. Silencing *AChE1* gene demonstrated significant mortality, developmental disruption, and heightened susceptibility to insecticides, underscoring its central role in pest physiology and neurotransmission. Similarly, *SEC23* gene silencing induced increased mortality, deformities, and reduced transcript levels, emphasizing its vital contribution to insect viability. The promising outcomes of this study not only highlight the genes' roles in insect survival but also hold promise for sustainable pest management strategies, contributing to the advancement of environmentally friendly approaches in agriculture. Further exploration of these genes may reveal opportunities to develop RNAi-based pest control methods, enhancing their efficacy and promoting sustainable agricultural practices.

CRediT authorship contribution statement

Muneeb Hassan Hashmi: Writing – original draft, Methodology, Investigation. **Haneef Tariq:** Writing – review & editing, Writing – original draft, Software, Investigation. **Faisal Saeed:** Methodology. **Ufuk Demirel:** Supervision. **Ayhan Gökçe:** Software, Investigation. **Hans Merzendorfer:** Supervision, Resources. **Emre Aksoy:** Writing – review & editing, Methodology, Investigation. **Allah Bakhsh:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100569.

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