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Validamycin affects the development and chitin metabolism in *Spodoptera frugiperda* by inhibiting trehalase activity

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With 5 figures

Abstract: The fall armyworm (FAW) *Spodoptera frugiperda* is a major polyphagous pest that feeds mainly on food crops, such as corn and rice, posing a serious threat to the safety of food crops worldwide. Trehalase, can degrade trehalose and the resulting products participate in the synthesis of chitin, one of the main components of outer epidermis in insects. Therefore, trehalase inhibitors have been identified as novel agents for pest control. We report the insecticidal potential of a trehalase inhibitor validamycin against FAW. Validamycin (1 μg/μL) when injected in FAW using microinjection could significantly reduce trehalase activity 48 h later, especially, the activity of soluble trehalase, then the glucose content and glycogen content also decrease. In addition, the deaths of larvae in validamycin treated group at each instar stage were higher than those in the control group, moreover, validamycin treatment increased the pupal weight and prolonged pest development. Similarly, validamycin also significantly inhibited the chitinase activity and caused the accumulation of chitin in FAW. Results of qRT-PCR showed that the expression levels of two trehalose genes (*SfTRE1* and *SfTRE2*), which encode key enzymes in chitin biosynthesis pathway, were significantly reduced, while the expression levels of fructose-6-phosphate transaminase, glucosamine-phosphate N-acetyltransferase and chitinase genes were significantly increased. It is obvious that validamycin has an influence on physiological metabolism and development of FAW, the results obtained will be conducive to the prevention and control of FAW in the future.

Keywords: Trehalase inhibitor, chitinase, glucose, fall armyworm, biopesticide

1 Introduction

Trehalose is a non-reducing disaccharide composed of two glucose units combined by α-1,1-α-link, present in various organisms, such as bacteria, fungi, plants, and invertebrates (nematodes and insects), but is absent in mammal (Elbein et al. 2003). In insects, Trehalose is synthesized in the fat body by the Trehalose-6-phosphate synthase (TPS)/ Trehalose-6-phosphate phosphatase (TPP) biosynthesis pathway (Shukla et al. 2015), and is quickly released into lymph nodes and other tissues. Trehalase is the only enzyme that hydrolyses trehalose irreversibly, and two different forms of trehalase exist in insects, soluble trehalase (TRE1) and membrane-bound trehalase (TRE2). TRE1 and TRE2 are genetically homologous but perform different functions. TRE1 is overexpressed in the corneum, midgut, and Malpighian tubule (De Almeida et al. 2009). In contrast, TRE2 is a transmembrane enzyme whose active site is located outside the cell membrane and is mainly highly expressed in the fat body, flight muscles, cuticle, midgut, and Malpighian tubule of insects (Silva et al. 2009). Trehalose can be hydrolyzed into two glucose molecules, making trehalose the main storage form of carbohydrates in insects, and is involved in the regulation of insect growth,

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flight, and tissue-protection (Wang et al. 2020; Shen et al. 2021).

Trehalase can regulate also the expression of chitin biosynthesis-related genes in insects (Tang et al. 2017). The chitin biosynthesis pathway began in trehalose decomposition, and requires the involvement of eight enzymes, besides trehalase, namely, hexokinase (HK), glucose 6-phosphate isomerase (G6PI), fructose-6-phosphate transaminase (GFAT), glucosamine-phosphate N-acetyltransferase (GNA), phosphoracetylglucosamine mutase (PAGM), and UDP-N-acetylglucosamine pyrophosphorylase (UAP), chitin synthase (CHS) (Zhu et al. 2008). The deletion of key chitin biosynthesis pathway genes may affect insect survival and growth, e.g. silencing *UAP* led to >50% mortality in *Bactrocera dorsalis* and to larvae showing abnormal phenotypes (Yang et al. 2015), and *TRE* knockout can lead to high mortality of *B. dorsalis* (Ye et al. 2019). In addition, the degradation of chitin also affects insect development. Chitin is the main component of the cuticular layer, which forms the insect exoskeleton and the physical and chemical barrier that protects the insects from dehydration, mechanical damage, and predation (Muthukrishnan et al. 2020). *CHT* knockout or inhibition of chitinase produces problems in ecdysis in various insects (Chen et al. 2019).

As a key hydrolase linking these two pathways, trehalase has been identified as a novel target specific for pests, as it does not affect vertebrates (Elbein et al. 2003). Several analogues targeting trehalase have been proposed as insecticides due to its critical role in insect physiology. Validamycin is considered a promising competitive trehalase inhibitor, effectively inhibiting trehalase in pests and resulting in developmental defects. The inhibition of trehalase is fatal for many pests due to hampered ecdysis and/or lack of chitin formation (Shukla et al. 2015). In the era of the green economy, trehalase inhibitors such as validamycin may become valuable for developing more selective and safer pesticides.

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is major polyphagous lepidopteran pest in the world (Kenis et al. 2022), and its management mainly relies on chemical pesticides and genetically modified crops (such as Bt maize) (Kenis et al. 2022). However, extensive use of chemicals in crops could negatively impact non-target organisms (Desneux et al. 2007), as well as could lead to development of resistance to different insecticides (Paula et al. 2021; Wang et al. 2021a). Environment friendly and sustainable pest control strategies are therefore needed. Although validamycin has been investigated in inhibiting some fungi and pests, its use has rarely been reported for the management of lepidopteran insects. In this context we investigated the effects of validamycin on trehalose metabolism and the chitin biosynthesis pathway in FAW and explored whether validamycin can inhibit FAW growth and development.

2 Material and methods

2.1 Insects and reagents

The FAW colony was provided by the Zhejiang Academy of Agricultural Sciences (Hangzhou, China) and individuals were reared at Hangzhou Normal University. Adults were fed with 10% honey water to lay eggs, which were collected for hatching, and the newborn larvae were fed with an artificial diet. The 2nd instar larvae were picked and fed artificial diet in a 4.5 cm diameter and 2.5 cm height little feeding box. Adults and larvae were cultured in a chamber under controlled conditions of 26 ± 1 °C temperature, 16 h/8 h (day/night) photoperiod, and 60 ± 10% relative humidity. In the study, larvae on the first day of the 3rd instar were used for validamycin injection (based on Cruz et al. 2017) and the artificial food formula (Sup. Table 1) was based from the study by Pinto et al. (2019). Validamycin (C178990, Lot: 20306) was obtained from Dr. Ehrenxtorfer and prepared in Germany. Validamycin was diluted with deionized water to obtain different concentrations.

2.2 Injection of validamycin

Based on the studies by Tang et al. (2017) and Yu et al. (2021a), five different concentrations of validamycin (0.5, 1, 2.5, 5, and 10 μg/μL) were prepared for this study. Using the NARISHIGE IM-31 microinjector (Nikon, TOKYO, JAPAN), different validamycin concentrations were injected between the second and third thoracic feet of each FAW specimen (validamycin group), and 300 nL validamycin was injected per pest. The untreated larvae were used as a control group (CK). Samples were collected 48 h after injection for subsequent analyses. The effective concentration was used in subsequent experiments.

2.3 Determination of trehalase and chitinase activities

Ten individual insects collected after injection were homogenized with stainless steel balls and then mixed with 1 mL phosphate-buffered saline (PBS, pH 7.0). This was followed by 30 min sonication (VCX 130PB, Sonics, Newtown, CT, USA). The samples were centrifuged at 1,000 × *g* at 4 °C for 20 min. Subsequently, 350 μL supernatant was collected and centrifuged again for 60 min at 20,800 × *g* and 4 °C, 300 μL supernatant was collected and used to determine the activity of soluble trehalase and protein concentration. The precipitates were suspended in 300 μL PBS and used to determine the membrane-bound trehalase activity and protein concentration. Next, the supernatant and suspension (60 μL) were mixed separately with 75 μL of 40 mM trehalose (Sigma-Aldrich, Saint Louis, MO, USA) and PBS (165 μL). The mixture was incubated at 37 °C for 60 min and inactivated by 5 min incubation at 100 °C. The trehalase activity was measured using the glucose (Go) Assay Kit (Sigma-Aldrich), and

the reaction was terminated by adding 12 N H_2SO_4 (260 μL). Finally, the absorbance at 540 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The protein contents of samples were determined using the BCA Protein Assay Kit (Beyotime, China).

Chitin broken down into N-Acetyl glucosamine by chitinase, furthering reacted with DNS (3,5-dinitrosalicylic acid) reagent to produce a brown-red compound, which has a characteristic absorption peak in 540 nm. Using the Chitinase Kit (Suzhou Comin Biotechnology Co., Ltd., China) according to the manufacturer's instructions. 0.1 g tissues were homogenized with 1 mL extracting solution to make crude enzyme solution which were centrifuged at 10,000 × *g* at 4 °C for 20 min, 400 μL supernatant were collected to control tube and the assay tube respectively, then added 600 μL of extracting solution to the control tube and 200 μL of the extracting solution and 400 μL of Reagent 1 to the assay tube. The mixture was incubated at 37 °C for 60 min and inactivated by 5 min incubation at 100 °C, then centrifuged at 10,000 × *g* at 4 °C for 20 min. 700 μL supernatant were collected to control tube and the assay tube respectively and added 500 μL Reagent 2 to each, incubated for 5 min at 95 °C in a water bath, the chitinase activity was measured at 540 nm. Chitinase activity (mg/h/g fresh weight) = 3.899 ×(ΔA+0.2753) / W (A, absorbance value; ΔA=A assay -A control; W, sample weight).

2.4 Determination of carbohydrate content

In this study, 1 mL PBS was added to the samples after homogenization, followed by 30 min sonication, and 20 min centrifugation at 1,000 × *g* and 4 °C. The supernatant was used to measure the contents of trehalose and glycogen. The remaining supernatant uses ultracentrifuged for 60 min at 20,800 × *g* and 4 °C and then divided into supernatant and suspension (formed by adding 150 μL PBS to the precipitate) for measuring glucose content.

The amount of trehalose was detected using anthrone reagent. Briefly, 1% sulfuric acid (H_2SO_4, 30 μL) was added to the 30 μL sample, incubated for 10 min at 90 °C in a water bath, and 30% KOH (30 μL) was added after 3 min incubation in an ice bath. Developer (600 μL of 0.02 g anthrone in 100 mL 80% H_2SO_4) was added to a test tube containing the mixed solution, incubated in hot water (90 °C) for 10 min, then cooled on ice. The absorbance was measured at 630 nm. The glycogen and glucose were detected similarly; however, the sample of glycogen (160 μL) was first taken out and mixed with 32 μL of 0.1 U/L of amyloglucosidase (Sigma-Aldrich) in a 1.5 mL Eppendorf (EP) tube. It was converted to glucose by incubating at 40 °C in a water bath for 4 h. In the next step, the glucose (Go) Assay Kit was used to measure the levels of glucose, followed by the addition of 12 N H_2SO_4 (260 μL) to terminate the reaction. The absorbance was read at 540 nm.

2.5 Chitin content measure

The method was based on the previous study by Bolognesi et al. (2005). One FAW larva was washed with distilled water, ground after freezing in liquid nitrogen, and then suspended in 6% KOH solution (500 μL). To deacetylate chitin, the samples were incubated at 80 °C for 90 min. After 20 min centrifugation at 12,000 × *g* at 4 °C, the supernatant was removed, the precipitate was resuspended in PBS (1 mL), and centrifuged again for 20 min. Next, the supernatant was discarded, and the precipitate was resuspended in McIlvaine's buffer (200 μL of 0.1 mol/L citric acid and 0.2 mol/L NaH_2PO_2, pH = 6.0). To hydrolyze chitin, 5 μL chitinase from *Streptomyces griseus* (Sigma-Aldrich) was added and incubated at 37 °C for 72 h. When the reaction was completed, the sample was centrifuged at 12,000 × *g* for 1 min at 25 °C. The supernatant was obtained (60 μL), and an equal volume of sodium borate (0.27 mol/L) was added. The mixture was stirred and incubated in a water bath (99.9 °C) for 10 min. The mixture was cooled down to 25 °C, 1×DMAB (600 μL) was added, and the reaction was incubated at 37 °C for 20 min. Standard calibration curves were prepared from stocks (0.05–2.25 mM GlcNAc). The absorbance of the sample and standard solution was determined at 585 nm.

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of FAW was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by 1% agarose gel electrophoresis. The concentration and quality of the RNA extracted were determined using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Next, the cDNA was synthesized using PrimeScript® RT reagent Kit With gDNA Eraser (NARISHIGE, JAPAN) and the relative expression of key genes involved in the chitin biosynthesis pathway was analyzed using qRT-PCR.

Briefly, a 10 μL reaction volume contained 5 μL SYBR Green master mix (SYBR Green Premix Ex Taq, Takara, Japan), 3.2 μL deionized water, 0.4 μL each of forward primer and reverse primers (10 μmol/L), and 1 μL cDNA sample. Each experiment was carried out using three biological replicates and three technical replicates on Bio-Rad CFX96TM Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the following reactions conditions: 95 °C pre-denaturation for 30 s, 95 °C denaturation for 5 s, 60 °C extension for 20 s, and 40 cycles. The relative expression levels of the genes were analyzed using the $2^{-\Delta\Delta CT}$ method. The list and sequences of primers used in the study are shown in Sup. Table 2.

2.7 Statistical analyses

Data are expressed as mean ± standard error (SE) and were evaluated for normality and homogeneity of variance.

Statistical analysis was done using the SPSS Ver.20 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple range test was used to compare the differences between the treatment group and the control group, Student's *t*-test were used for independent samples. All experiments were performed in triplicate with three biological replicates and at least three technical replicates.

3 Results

3.1 Impact of validamycin on FAW trehalase activity

The trehalase activity in FAW 48 h after validamycin treatments was significantly different than in the control group (F = 180.834; df = 5, 12; P < 0.001, Fig. 1A) except for the 0.5 μg/μL concentration. All other concentrations drastically reduced the activity, with 10 μg/μL showing the significant highest reduction. The membrane-bound trehalose activity was also significantly reduced by validamycin at 1, 5 and 10 μg/μL (F = 34.874.; df = 5, 12; P < 0.001; Fig. 1B) whereas other concentrations did not differ from control (P > 0.05). Validamycin could inhibit the trehalose activity of FAW and the lowest effective concentration was 1 μg/μL.

3.2 Contents of trehalose, glucose, and glycogen after treatment with different concentrations of validamycin

Validamycin had no significant effect on trehalose content in FAW (Fig. 2A) when compared to the control group. However validamycin at 1 μg/μL significantly reduced the glucose and glycogen contents (t = 4.187; P = 0.014, t = 0.392; P = 0.042, respectively; Fig. 2B, 2C), and 10 μg/μL of validamycin significantly reduced glucose (t = 0.641; P = 0.021) (Fig. 2B). Other concentrations of validamycin had not effect on glucose and glycogen concentrations (all P > 0.05); the 1 μg/μL concentration was used in subsequent experiments as impacting FAW trehalose metabolism.

3.3 Death and phenotypes of FAWs

The survival and phenotypes of FAWs were observed every 24 h after injecting validamycin; FAW survival rate decreased sharply at the time of emergence in both groups, while the number of deaths before emergence was not significant in both groups (Fig. 3A). Interestingly insects from the control group started dying after the 5th instar whereas those in the validamycin group started dying earlier (at 4th instar). Mortality observed in FAWs in the validamycin group were related to molting issues, incomplete pupation, and/or the inability of adults to break pupae or wing deformity (Supplementary Fig. 1), although no significant difference was found in pupation (t = 0.779; P = 0.446) and emergence (t = –0.191; P = 0.855) between the validamycin and control groups (Fig. 3B).

3.4 Growth and development of FAWs

The developmental duration of each instar in the validamycin group was significantly longer than that in the control. The pre-pupa stage duration in the validamycin group was shorter than that in the control, however, the overall developmental duration was significantly longer than that of the control (t = –2.776; P = 0.009; Fig. 4A). However, the length of

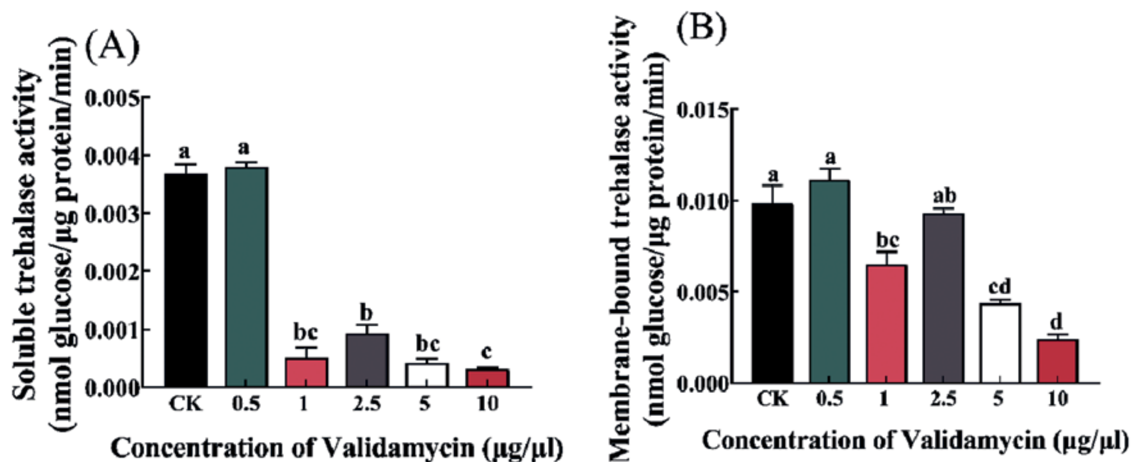

Fig. 1. Changes in the trehalase activity after 48 h treatment with five different concentrations of validamycin (CK: control). **(A)** change in the activity of soluble trehalase and **(B)** change in the activity of membrane-bound trehalase. Every group had three replicates (ten insects were used per replicate). Mean ± SE, and different letters within the same parameter differ significantly (P < 0.05, ANOVA followed by Tukey's post hoc test).

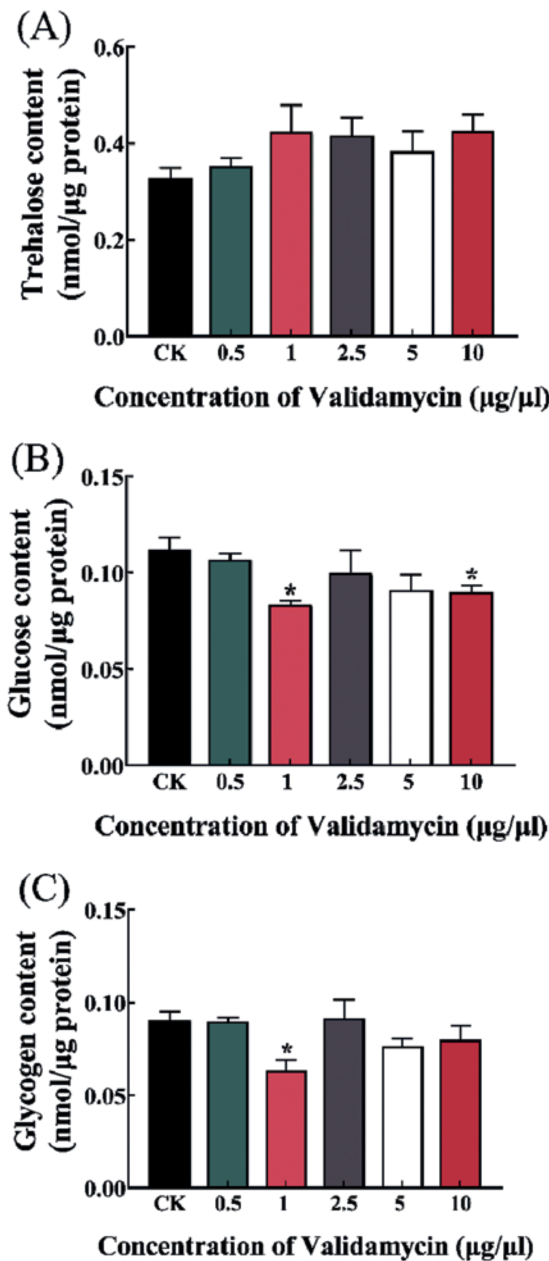

Fig. 2. Changes in trehalose content **(A)**, glucose content **(B)**, and glycogen content **(C)** after 48 h validamycin treatment (CK: control). Every group had three replicates (ten insects were used per replicate). Values are presented as the mean ± SE. *: $P < 0.05$ (independent-samples *t*-test).

4th, 5th and 6th instars as well as pupa did not differ between validamycin-treated and control (4th instar, t = 1.062; P = 0.294. 5th instar, t = –0.808; P = 0.423. 6th instar, t = 1.402; P = 0.167. pupa, t = 0.399; P = 0.691; Fig. 4B). Pupal weight was found significantly increased in the validamycin group (0.217 g ± 0.005) when compared to the control (0.194 g ± 0.003; t = –4.196; $P < 0.001$).

3.5 Changes of chitinase and content of chitin after validamycin treatment

Validamycin is a well-known trehalase inhibitor; however, it also inhibits chitinase. In our experiments, chitinase activity decreased by 52.7% in the validamycin group (6.720 ± 0.676 mg/h/g fresh weight) compared with the control group (14.521 ± 1.758 mg/h/g fresh weight) (t = 4.143; P = 0.014), suggesting that the chitinase activity was significantly reduced. Chitin content in validamycin group (0.153 ± 0.016 μg) increased significantly (58.9%, t = –2.975; P = 0.041) compared with the control (0.096 ± 0.018 μg).

3.6 Relative expression levels of key genes in the chitin biosynthesis pathway after validamycin treatment

The relative expression levels of genes related to chitin biosynthesis were analyzed using qRT-PCR after 48 h treatment with 1 μg/μL validamycin (Fig. 5). The relative expression levels of *SfTRE1* (t = 11.131; P = 0.010) and *SfTRE2* (t = 4.276; P = 0.013) were significantly lower than the control group (*SfTRE1* and *SfTRE2* were 0.148 times and 0.272 times of the control group). On the contrary, the relative expression levels of some genes involved in the chitin biosynthesis pathway were up-regulated, and the relative expression levels of *SfGFAT* (4.82 times of the control group, t = –3.279; P = 0.031), *SfGNPN* (22.35 times of the control group, t = –4.796; P = 0.017), and *SfCHT* (10.29 times of the control group, t = –3.434; P = 0.041) were up-regulated significantly. Expression levels of other genes (*SfPAGM, SfHK2, SfG6PI, SfUAP*, and *SfCHSB*) showed no significant changes.

4 Discussion

We used five different concentrations of validamycin for the microinjection of FAW and two trehalase activities were inhibited by the various validamycin concentrations but 0.5 μg/μL; the lowest observed effect concentration being 1 μg/μL. This validamycin concentration was found to reduce the glucose content and glycogen content in FAWs. The trehalose activity was inhibited and cannot degrade trehalose, which resulted in the reduction of glucose levels. With the decrease of glucose content, which is an intermediate metabolite for the synthesis of glycogen (Shi et al. 2016), the glycogen synthesis was also relatively reduced. Interestingly, the highest concentration of validamycin (10 μg/μL) reduced glucose content but had no effect on the glycogen content. Glycogen is synthesized from UDP-glucose via glycogen synthase (GS) (Zhang et al. 2017). UDP-glucose mainly derived from dietary carbohydrates and glucose (Thompson 2003). Inhibiting trehalase activity will cause trehalose is rarely converted to glucose, and glucose has been consumed by organism's metabolism furthering decrease the glucose content. Trehalose is accumulated because of its decomposed rarely so that GS synthesizes UDP-glucose to glyco-

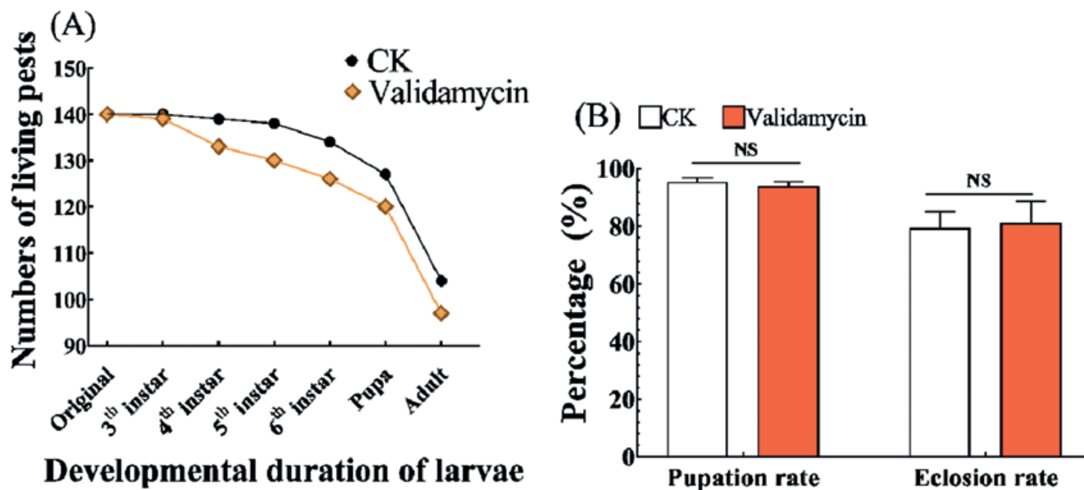

Fig. 3. Death number and phenotype of FAW larvae after 1 μg/μL validamycin injection (CK: control). Recorded number of deaths from 24 h after validamycin treatment to adult eclosion **(A)**; the pupation rate and eclosion rate of FAW during development **(B)**. Pupation rate = (pupal number /6th instar larval number) * 100%; Eclosion rate = (number of adults/pupal number) * 100%. N = 140–180 per treatment. Values are presented as the mean ± SE. NS: not significant (independent-samples t-test).

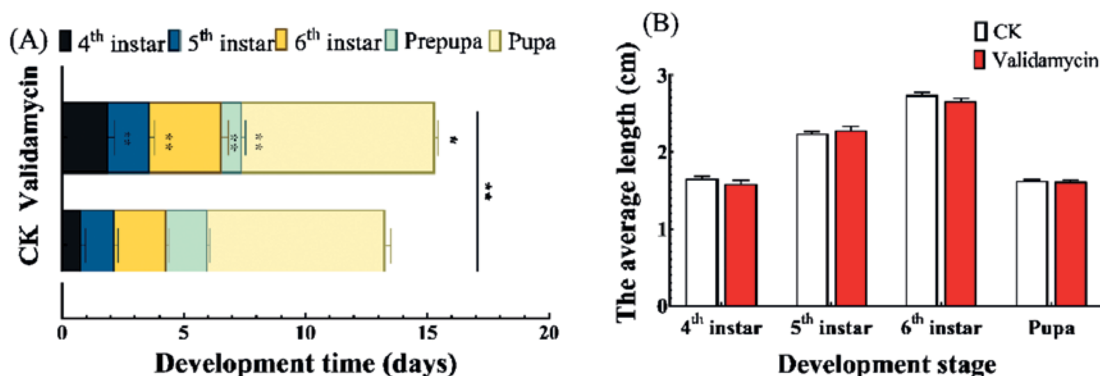

Fig. 4. Duration of development in *S. frugiperda* larvae after treatment with validamycin (CK: control). The growth time of each instar and the time required for 4th instar larvae-emergence **(A)**. Larval length of each instar **(B)**. Every group had three replicates (fifty insects were used per replicate). Values are presented as the mean ± SE. *: $P < 0.05$, **: $P < 0.01$ (independent-samples t-test).

gen. FAWs might be feeding as possible when stimulated in high validamycin concentration (10 μg/μL) to promote glycogen synthesis which compensated for the reduced glycogen caused by a few glucoses. In the future, aspects such as feeding, and GS activity can be researched to explain this phenomenon. In vivo experiments, Wegener et al. (2010) indicated trehalase differentiates between an "overt" and a "latent" trehalase. Membrane-bound trehalase activity that can be measured directly in tissue homogenates and soluble trehalase activity are designated as overt trehalase activity. trehalase activity is increased after the disruption of membrane integrity by detergents, this additional activity is called latent trehalase activity. Latent activity could be interpreted as a precursor or a storage form of trehalase activity that was not initially exposed to the inhibitor, but was later transformed into overt trehalase. The membrane-bound isoform of trehalase (trehalase-2) is believed to be the latent (inactive) form, more abundant in larvae (Forcella et al. 2010). Validamycin treatment for 12 h and 24 h inhibited soluble trehalase activity invariably, but treatment for 24 h the membrane-bound trehalase activity was increased in *Spodoptera litura* (Yu et al. 2021b). In our previous experiments, 2.5 μg/μL validamycin concentration always had no obvious inhibitory effect on membrane-bound trehalase activity. Yu's

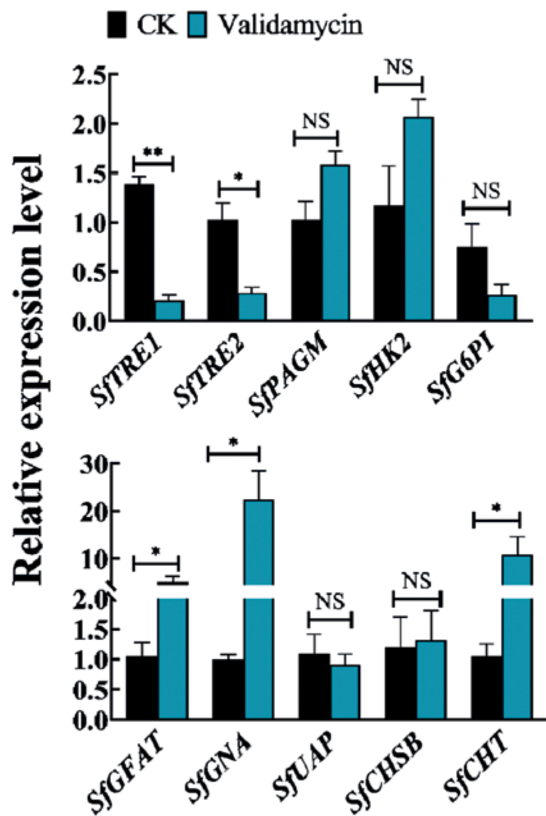

Fig. 5. The relative expression levels of genes related to trehalose metabolism and chitin biosynthesis in *S. frugiperda* larvae after 48 h validamycin (1 μg/μL) treatment (CK: control). Expression of two trehalose metabolism pathway genes (*SfTRE1* and *SfTRE1*), seven chitin biosynthesis pathway genes (*SfPAGM, SfHK2, SfG6PI, SfGFAT, SfGNA, SfUAP, SfCHSB*), and one chitin gene (*SfCHT*) after 48 h. TRE1, soluble trehalase; TRE2, membrane-bound trehalase; PAGM, phosphoracetylglucosamine mutase; HK2, hexokinase 2; G6PI, glucose-6-phosphate isomerase; GFAT, fructose-6-phosphate transaminase; GNA, glucosamine-phosphate N-acetyltransferase; UAP, UDP-N-acetylglucosamine pyrophosphorylase; CHSB, chitin synthase B; CHT, chitinase. Every group had three replicates (five insects were used per replicate). Mean ± SE. *: $P < 0.05$, **: $P < 0.01$, NS: not significant (independent-samples *t*-test).

results and our results shown that validamycin has stable inhibition of soluble trehalase, increased membrane-bound trehalase may probably activated after "latent", it may be the reason why that membrane-bound trehalose activity was not reduced by the 2.5 μg/μL validamycin concentration. However, scarce information is available about membrane-bound trehalase of insects, the phenomenon should be further explored.

When the expression levels of *SfTRE1* and *SfTRE2* were inhibited by 1 μg/μL validamycin, *SfGFAT, SfGNA,* and *SfCHT* expression levels were upregulated. Kato et al. (2002) indicated that GFAT and GNA play a regulatory role in chitin biosynthesis. Similarly, when shrimp *Litopenaeus vannamei* is exposed to alkaline pH and cadmium stress, *LvGFAT* is strongly expressed in the liver, suggesting that this gene plays an important role in the environmental stress tolerance (Liu et al. 2015). After organism was stimulated by decreasing trehalase the upregulation of *SfGFAT* and *SfGNA* expression are likely to be the feedback mechanism to maintain chitin biosynthesis. Bolognesi et al. (2005) reported that *SfCHSB* and *SfCHI* (here represented by *SfCHT*) are over-expressed in midgut epithelium. There is little or no chitin in the midgut when *SfCHI* is expressed, and this shows that *SfCHI* is mainly responsible for chitin degradation in peritrophic membrane (PM) (Bolognesi et al. 2005). Defective PM disrupts the digestive enzyme cycle in the midgut of FAW larvae and increases mortality in *Trichoplusia ni* larvae (Bolognesi et al. 2001; Wang et al. 2001). Overexpression of *SfCHT* occurred 48 h after validamycin treatment, an important stage in the development of FAW from the 3rd instar to the 4th instar. It may explain gradual reduced number of surviving FAW after 4th instar in validamycin group (Fig. 3A).

We observed that chitin content of FAW after the trehalase activity was strongly inhibited, and surprisingly the chitin content was increased in the validamycin group compared to the control group, whereas, contrary to our results, the chitin content reportedly reduced after *TRE* (*Acyrthosiphon pisum, Nilaparvata lugens*) knockout in earlier studies (Zhang et al. 2017; Wang et al. 2021b). We detected a strong reduction in the chitinase activity of FAW confirming the strong chitinase inhibitory effect of validamycin. Most of *GpCHT* reduced at 24 h and increased at 48 h after injection with Validamycin A in *Glyphodes pyloalis* (Shao et al. 2021), which was an interesting phenomenon for us because *SfCHT* expression was also increased at 48 h. We hypothesize that it may be that cells promote transcription to maintain homeostasis. Yu et al. (2021) considered that validamycin treatment in the late stage (12–24 h) to compensate for chitin deficiency in *S. litura* the genes were further related to chitin synthesis activated to synthase a large amount of chitin. Both "later stage" (48 h after treatment) of chitin content detection and results of chitin synthesis related gene expression level agree with Yu et al. (2021b), it further confirms our hypothesis. Therefore, it is reasonable to think that the difference in chitin content from earlier studies may be the difference in tolerance of different species to validamycin, finally result in a bias in the time of cell stress. During insect development and metamorphosis, the fluctuation of chitin content depends on the activities of chitin synthase and chitinase (Zhu et al. 2016). Insects have more chitinase than other organisms, and their chitinase is produced for the exfoliation of the old cuticle during ecdysis. In recent years, catalytic and structural characterization studies of *OfChtI* from *Ostrinia furnacalis* have shown that chitinase is essential for molting in lepidopterai (Chen et al. 2014). Therefore, it is reasonable to conclude that the accumulation of chitin was the results

of a combination of reduced chitinase activity and feedback regulatory mechanism.

The decrease in trehalase or chitinase activity has a significant impact on the survival and normal development of insects. In our study, the deaths of FAWs in the validamycin group were higher than that in the control group. In addition, abnormal ecdysis occurred at all instar stages. Studies by Tang et al. (2017) and Yu et al. (2021a) demonstrated that *N.lugen* and *Diaphorina Citri* both suffered extremely high mortality due to abnormal ecdysis after treatment with validamycin. In addition, the survival rate of *Helicoverpa armigera* reduced by 10–20% after 10 days of feeding with validamycin A; moreover, feeding with validamycin A resulted in various developmental defects and adverse effects on insect ecdysis and metamorphosis, with malformation observed in 10–15% of pupa along with delayed emergency (Adhav et al. 2018). Our results are consistent with those of previous studies. Trehalase converts the insect trehalose into glucose, which is then used to release energy (Shukla et al. 2015). Validamycin injection reduced the energy supply by decreasing glucose content in FAW, which resulted in longer development period at each FAW instar in the validamycin group than in the control group. The pupal weight in the validamycin group was abnormally increased, hinting that further studies are needed to depict mechanisms at play in observed effects.

We showed that validamycin could inhibit both trehalase and chitinase activities. We hypothesized that inhibiting trehalase activity might reduce the glucose content in insects resulting in a shortage of energy supply and thus prolonging the development of insects. In contrast, the downregulation of chitinase activity led to the accumulation of chitin, resulting in the inability of insects to shed the old outer epidermis and eventually death. Our study find that validamycin can block chitinase and the degradation of chitin, providing a novel strategy for controlling diseases and insect pests. However, the mechanisms underneath the reduced chitinase activity will require further investigations.

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