



# Physiological Alterations in Deletion Mutants of Two Insulin-Like Peptides Encoded in *Maruca vitrata* Using CRISPR/Cas9

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Most insect species encode multiple insulin-like peptides (ILPs) that exhibit functional overlaps in mediating physiological processes such as development and reproduction. Why do they need multiple ILPs? To address this question, we tested a hypothesis of the requirement of multiple ILPs by generating mutants lacking individual ILP genes using the CRISPR/Cas9 technology. Two ILPs (ILP1 and ILP2) in the legume pod borer, *Maruca vitrata*, mediate similar physiological processes such as hemolymph sugar level, larval development, and adult reproduction. Individual knock-out mutants ( $\Delta$ ILP1 and  $\Delta$ ILP2) were generated. They showed successful development from larvae to adults. However, they suffered from high hemolymph sugar levels by enhancing trehalose titers in the hemolymph. The hyperglycemic effect was more evident in  $\Delta$ ILP2 mutants than in  $\Delta$ ILP1 mutants. Both mutants showed increased expression of *trehalose-6-phosphate synthase* but suppressed expression of *trehalase*. These mutants also showed altered expression patterns of insulin signaling components. Expression levels of *insulin receptor* and *Akt* genes were upregulated, while those of *FOXO* and *Target of rapamycin* genes were downregulated in these mutants. These alterations of signal components resulted in significant retardation of immature development and reduced body sizes.  $\Delta$ ILP1 or  $\Delta$ ILP2 females exhibited poor oocyte development. Bromouridine incorporation was much reduced at the germarium of ovarioles of these mutants compared with wild females. Expression of the *vitellogenin* gene was also reduced in these mutants. Furthermore, males of these deletion mutants showed impaired reproductive activities when they mated with wild-type females. These results suggest that both ILPs are required for mediating larval development and adult reproduction in *M. vitrata*.

**Keywords:** insulin-like peptide, trehalose, growth, reproduction, CRISPR/Ca9

## INTRODUCTION

Insect growth depends on favorable temperatures with a supply of nutrients. Favorable environmental cues promote immature growth through internal endocrine signals, mostly via insulin-like peptides (ILPs) (Okamoto and Yamanaka, 2015). In adult insects, ILPs can also mediate reproductive processes such as oogenesis and spermatogenesis (Ueishi et al., 2009; Lenaerts et al., 2019).

Insulin-like peptides encode a characteristic molecular structure containing a signal peptide, an A-chain, a C-chain, and a B-chain. After removing the C-chain via post-translational modification, a mature heterodimeric peptide consisting of A-B chains linked through a disulfide bond is produced and secreted in response to nutrient signals (Nassel and Vanden Broeck, 2016). The fat body is the central tissue that connects nutrients to ILP secretion from median neurosecretory cells in *Drosophila* (Rullifson et al., 2002) via a fat body-derived leptin-like protein called Unpaired 2 or a small peptide called CCHamide-2 in response to high fat or a high sugar diet (Rajan and Perrimon, 2012; Sano et al., 2015).

In target cells, signal transduction pathways mediated by insect ILPs are highly conserved among metazoans (Nijhout and Callier, 2013). Insect ILPs can use a transmembrane receptor tyrosine kinase called insulin receptor (InR) to activate both Ras-MAPK (mitogen-activated protein kinase) and PI3K/PKB (phosphatidylinositol-3-kinase/protein kinase B) pathways (Badisco et al., 2013). Once ILP binds to InR, it triggers the downstream ILP/insulin-like growth factor signal (IIS), beginning with autophosphorylation of InR, which then phosphorylates insulin receptor substrate (INS). Next, the phosphorylated INS can recruit PI3K to the membrane, where it phosphorylates phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-3,4,5-trisphosphate (PIP3). Accumulated PIP3 levels can then recruit phosphoinositide-dependent kinase (PDK), activating serine-threonine protein kinase (Akt). Akt then phosphorylates Forkhead Box O (FOXO) to prevent its translocation into the nucleus while phosphorylating the tuberous sclerosis complex to upregulate protein translation. Finally, the amino acid-mediated TOR (target of rapamycin) pathway independently activates the downstream signal of IIS, which promotes larval growth and adult reproduction (Das and Arur, 2017).

The first ILP was a small prothoracicotrophic hormone of *Bombyx mori* (silkworm) known to be bombyxin (Nagasawa et al., 1984). Since then, several ILPs have been identified in each insect species. Genome analysis has revealed that each insect species possesses multiple ILPs: four in *Tribolium castaneum* (red flour beetle), seven in *Anopheles gambiae* (African malaria mosquito), eight in *Drosophila melanogaster* (fruit fly), 10 in *Acyrtosiphon pisum* (pea aphid), and 39 in *B. mori* (silkworm) (Wu and Brown, 2006). These multiple ILPs can mediate specific physiological processes. For example, in *Drosophila*, ILP6 mediates insect growth while ILP7 and ILP8 mediate reproduction (Veenstra, 2020). However, there are functional overlaps among different ILPs. Currently, it is unclear why each insect has multiple ILP signals to mediate a specific physiological process.

The legume pod borer, *Maruca vitrata* (Lepidoptera: Crambidae), is an insect pest infesting leaves, flowers, and pods of leguminous crops in subtropical and tropical regions (Sharma, 1998). At least two ILPs (*Mv-ILP1* and *Mv-ILP2*) are known in this species. They play crucial roles in mediating larval development and adult reproduction (Al Baki et al., 2019a,b). Trehalose, a main hemolymph sugar, fluctuates in its titer depending on the feeding rhythm. Therefore, it is regulated

by both ILPs in *M. vitrata* (Al Baki et al., 2018a). These two ILPs also mediate oocyte development by stimulating mitotic division of stem cells and promoting vitellogenin (Vg) synthesis in *M. vitrata* (Al Baki et al., 2019b). Moreover, these two ILPs are similarly expressed in most developmental stages and different tissues (Al Baki et al., 2020). Thus, the functional redundancy of these two ILPs has been questioned, raising the physiological significance of multiple ILPs in this species.

Thus, the objective of this study was to clarify multiple ILPs in *M. vitrata* exhibiting functional overlap using individual deletion mutants against different ILPs with the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology.

## MATERIALS AND METHODS

### Insect Rearing

Larvae of *M. vitrata* were reared with an artificial diet (Jung et al., 2009) under the following laboratory conditions: temperature, 25 ± 1°C; photoperiod, 16:8 h (L:D), and relative humidity, 60 ± 10%. Under such conditions, larvae of *M. vitrata* underwent five instars (L1–L5). Adults of *M. vitrata* were fed with 10% sucrose. L5 larvae of *M. vitrata* aged by days after final larval molt from day 1 to day 5 (L5D1–L5D5). For oviposition, kitchen paper towels were provided in adult cages.

### CRISPR/Cas9—Preparation of Single-Stranded Guide RNA (sgRNA)

Genomic DNA structures of *Mv-ILP1* and *Mv-ILP2* were analyzed by sequencing its open reading frame using genomic DNA (gDNA). The resulting exon sequences of *Mv-ILP1* and *Mv-ILP2* were submitted to an online design tool<sup>1</sup> to detect the best target site. We selected 23 bp sgRNA containing protospacer adjacent motif (PAM) in each of *Mv-ILP1* and *Mv-ILP2*. A customized sgRNA was generated using a Guide-it sgRNA *In Vitro* Transcription kit (Takara Korea Biomedical, Seoul, South Korea) following two template DNA PCR amplification steps and consecutive *in vitro* transcription. Briefly, PCR was performed with a customer-designed forward primer [58 nucleotides = additional four nucleotides + T7 sequence (17 nucleotides) + target sequence (20 nucleotides) + two Gs + annealing site (15 nucleotides)] and a company-prepared reverse primer. The sgRNA was synthesized *in vitro* using T7 RNA polymerase according to the instructions of the manufacturer. The final amount of sgRNA was quantified with a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Wilmington, DE, United States) and purified using a spin column provided with the kit.

### CRISPR/Cas9—Microinjection of sgRNA and Cas9 Protein

After female adults of *M. vitrata* have laid eggs on kitchen paper towels for 1 h at the scotophase (3–4 AM), eggs were dried

<sup>1</sup><http://chopchop.cbu.uib.no>

in air for 10 min in a desiccator at room temperature (RT) and fixed on a cover glass. Sharp pointed ( $< 20 \mu\text{m}$  diameter) glass capillaries (10  $\mu\text{L}$  quartz, World Precision Instrument, Sarasota, FL, United States) were prepared with a Narishige magnetic glass microelectrode horizontal puller (model PN30, Triton Research, Los Angeles, CA, United States) for injection. Collected eggs were injected with microcapillaries using a sutter  $\text{CO}_2$ -based pico pump injector (PV830, World Precision Instrument) under a stereomicroscope (SZX-ILLK200, Olympus, Tokyo, Japan). Injection (5 nL/egg) was performed using a mixture containing Cas9 (500 ng/ $\mu\text{L}$ ) and sgRNA (50 ng/ $\mu\text{L}$ ) through a micropyle. All injections were accomplished within 30 min after egg collection, including drying time. Treated eggs were then incubated at RT for 4 h before they were transferred to a growing chamber (25°C) for hatching.

### gDNA Extraction and Identification of Mutagenesis

Genomic DNA was extracted from the hemolymph of newly hatched L5 larvae with Chelax (Bio-Rad, Hercules, CA, United States). To extract gDNA, 30  $\mu\text{L}$  hemolymph was mixed with 500  $\mu\text{L}$  of 20% Chelex and heated at 100°C for 10 min. After cooling on ice for 2 min, the suspension was centrifuged at  $10,000 \times g$  for 3 min. The resulting supernatant was used as a gDNA sample. PCR was performed using 80 ng of gDNA as a template to amplify the region containing the CRISPR target site. PCR product was then cloned into a TOPO-2.1 vector (Invitrogen, Carlsbad, CA, United States) and sequenced. Primers used for mutagenesis analysis are listed in **Supplementary Table 1**.

### RNA Extraction and cDNA Preparation

Each L5 larva was used for total RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the instructions of the manufacturer. First, extracted RNA was resuspended in nuclease-free water and quantified using a spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE, United States). Next, RNA (1  $\mu\text{g}$ ) was used for cDNA synthesis with RT PreMix (Intron Biotechnology, Seoul, South Korea) containing oligo dT primer according to the instructions of the manufacturer.

### RT-PCR and RT-qPCR

RT-PCR was performed using DNA Taq polymerase (GeneALL, Seoul, South Korea) under the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 52–58°C (**Supplementary Table 1**) for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min with gene-specific primers (**Supplementary Table 1**). Each RT-PCR reaction mixture (25  $\mu\text{L}$ ) consisted of cDNA template, dNTP (each 2.5 mM), 10 pmol of each forward and reversed primer, and Taq polymerase (2.5 unit/ $\mu\text{L}$ ). In addition,  $\beta$ -actin (**Supplementary Table 1**) was used as a reference gene because of its relatively stable expression in different tissues of *M. vitrata* (Chang and Ramasamy, 2014).

All gene expression levels in this study were determined using a real-time PCR machine (Step One Plus Real-Time PCR System, Applied Biosystem, Singapore) following the guideline of Bustin et al. (2009). RT-qPCR was performed in a 20  $\mu\text{L}$  reaction volume containing 10  $\mu\text{L}$  of Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA), 5  $\mu\text{L}$  of cDNA template (50 ng), and each 1  $\mu\text{L}$  of forward and reverse primers (**Supplementary Table 1**). RT-qPCR cycling began with heat treatment at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 52–58°C (**Supplementary Table 1**) for 30 s, and extension at 72°C for 40 s. The expression level of  $\beta$ -actin as an internal control was used to normalize target gene expression levels under different treatments. PCR products were assessed by melting curve analysis. Quantitative analysis was performed using the comparative Ct ( $2^{-\Delta\Delta\text{Ct}}$ ) method (Livak and Schmittgen, 2001).

### Hemolymph Collection and Trehalose Measurement Using HPLC

Hemolymph was collected after cutting the first abdominal prolegs of mutant L5 larvae. A few phenylthiourea (Sigma-Aldrich Korea, Seoul, South Korea) granules were added to the hemolymph sample to prevent coagulation. After centrifugation at  $400 \times g$  for 3 min, the supernatant plasma was diluted 20 $\times$  with distilled water. The diluted plasma sample was cleaned with a Sep-Pak C18 cartridge (Walters Associates, Milford, MA, United States). Trehalose was identified and quantified using high-performance liquid chromatography (HPLC) (BioLC, Dionex, Sunnyvale, CA, United States) with the main column (CarboPacMA1, 4  $\times$  250 mm, Dionex) and a guard column (CarboPacMA1, 4  $\times$  50 mm, Dionex) following the method described by Park and Kim (2013). The injection volume of the sample was 25  $\mu\text{L}$ . NaOH (400 mM) was used as an elution buffer at a constant rate of 4 mL/min. Pulse amperometry mode of an electrochemical detector (ED40, Dionex) was used for detecting trehalose and other sugars.

### *In vitro* Organ Culture and Bromouridine (BrdU) Incorporation

For *in vitro* organ culture, 5-day-old virgin females were used. Ovaries were collected and cultured in a TC-100 insect cell culture medium (Hyclone, Daegu, South Korea) containing 10  $\mu\text{M}$  BrdU (Sigma-Aldrich, Seoul, South Korea) for 24 h at 25°C. These ovaries were then fixed with 3.7% paraformaldehyde in a wet chamber under darkness at 25°C for 60 min. After washing with 100 mM phosphate-buffered saline (PBS, pH 7.4), ovarioles were permeabilized with 2% Triton X-100 in PBS at RT for 20 min. These ovaries were then rinsed with PBS three times, blocked with 5% skim milk (MB cell, Seoul, South Korea) in PBS at RT for 1 h, washed once with PBS, and incubated with mouse anti-BrdU antibody (BD Bioscience, San Jose, CA) diluted 1:15 in blocking solution for 1 h. Unbound anti-BrdU was washed off with PBS three times. Ovaries were then incubated with FITC-conjugated anti-mouse antibody (Sigma-Aldrich, Gangnam-Gu, South Korea) diluted 1:300 in blocking solution at RT for 1 h. After washing off the unbound anti-mouse antibody with

PBS three times, stained ovarian cells were observed under a fluorescence microscope (DM2500, Leica, Wetzlar, Germany) at 200× magnification.

## Test for the Reproductive Activity of Mutant Males

After confirmation of deletion mutants during the L5 stage by gDNA sequencing, newly emerged mutant adults were separated and used to obtain inbred or hybrid lines by reciprocal matings with mutant or wild-type adults having the same age. Each cross used one male and two females. Total eggs laid by a single female and subsequent hatching rate were recorded. Each mating treatment was replicated three times.

## Statistical Analysis

All results are expressed as mean ± SD. They were plotted using Sigma Plot (Systat Software, San Jose, CA, United States). Means were compared by least square difference (LSD) test based on one-way analysis of variance using PROC GLM of SAS program (SAS Institute, 1989) and discriminated at Type I error = 0.05.

## RESULTS

### Generation of CRISPR Mutants Against Two ILP Genes

To confirm the physiological functions of two ILPs in the development and reproduction of *M. vitrata*, CRISPR/Cas9 mutagenesis technology was applied to generate two mutants with deletion of either *Mv-ILP1* or *Mv-ILP2* from its genome (Figure 1). To design single-stranded guide RNA (sgRNA), genome structures within their open reading frames (ORFs) were assessed by sequencing gDNA that showed no intron. sgRNA was designed in the size of 20-mer near the PAM motif (Figure 1A). Newly laid eggs (< 5 h) were injected with sgRNA and Cas9 protein through micro-capillaries in a 5 nL volume. For control eggs injected with the only sgRNA, the hatching rate was about 17.0% (Figure 1B). For eggs injected with sgRNA + Cas9 protein, the hatching rate was only 7.7% for *Mv-ILP1* or 7.5% for *Mv-ILP2*. All larvae developed into fifth instar larvae. Their genomes were assessed to determine the efficacy of CRISPR mutagenesis by sequencing the target region using forward (Fwd) and reverse (Rev) primers. CRISPR mutagenesis against *Mv-ILP1* and *Mv-ILP2* resulted in mutation rates of 45.8% and 42.4%, respectively. In addition, these mutants exhibited deletion mutation of nucleotide in the target region. Ten (M1–M10) and nine (M1–M9) different types of deletion mutants were generated in *Mv-ILP1* and *Mv-ILP2*, respectively (Figure 1C). Mutants ( $\Delta$ ILP1) in *Mv-ILP1* had deletions ranging from two nucleotides (M5) to 16 nucleotides (M6), in which four mutants (M4, M5, M6, and M10) had early termination signal by a subsequent frameshift mutation. Similarly, *Mv-ILP2* mutants ( $\Delta$ ILP2) had deletions ranging from two nucleotides (M2) to 17 nucleotides (M1), in which four mutants (M1, M2, M5, and M8) had early termination signal. All the deletion mutants were

used to obtain next-generation mutants in each ILP gene for subsequent bioassays to monitor physiological alterations.

### Alteration of Hemolymph Trehalose Levels in Two ILP Deletion Mutants

Trehalose was the major hemolymph sugar of wild or mutant larvae of *M. vitrata* when measured at 2 h after light-off (18 h after light-on, Figure 2A). Hemolymph trehalose levels were relatively constantly maintained at about 80 mM in control larvae, showing a slight fluctuation from 75 to 90 mM (Figure 2B). However, two ILP mutants had slightly higher trehalose levels during most photophase and increased significantly ( $P < 0.05$ ) at around light-off. At 2 h after light-off, trehalose level was about 120 mM in  $\Delta$ ILP1 and about 150 mM in  $\Delta$ ILP2 while control larvae kept the basal level of trehalose titer in the hemolymph.

To explain the hypertrehalosemia in two deletion mutants, expression levels of two genes associated with trehalose synthesis (trehalose-6-phosphate synthase: TPS) and degradation (trehalase: TRE) were assessed (Figure 3). These two genes were expressed in both wild-type and mutant larvae (Figure 3A). When RT-qPCR quantified their expression levels, both mutants showed significantly ( $P < 0.05$ ) increased TPS expression levels but downregulated TRE expression levels (Figure 3B). Especially,  $\Delta$ ILP2 mutants showed significantly more upregulation of TPS expression ( $P < 0.05$ ) than  $\Delta$ ILP1 mutants.

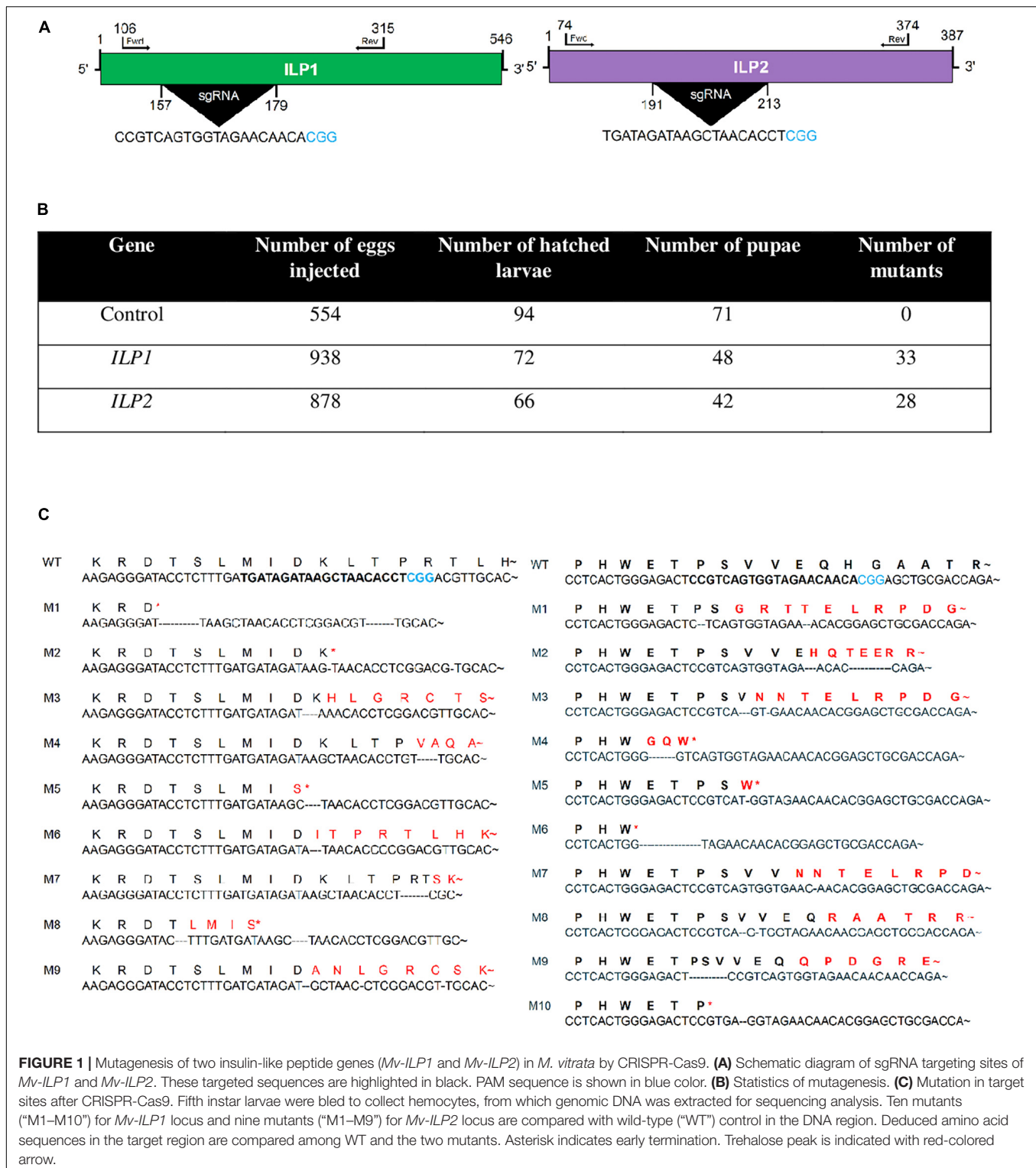
### Dysregulated Gene Expression of IIS Components in Two ILP Deletion Mutants

The lack of ILP in  $\Delta$ ILP1 and  $\Delta$ ILP2 mutants might change the expression profile of ILP signaling components. To test this hypothesis, expression levels of four IIS component genes were assessed by RT-qPCR (Figure 4). Compared with control larvae,  $\Delta$ ILP1 and  $\Delta$ ILP2 mutants exhibited almost fourfold and sixfold increases of *Mv-InR* expression levels. In addition, expression levels of *Mv-Akt* were increased almost 2.5-fold in these mutants. However, gene expression levels of *Mv-FOXO* and *Mv-TOR* were significantly ( $P < 0.05$ ) suppressed in these mutants. These supported the hypothesis of IIS dysregulation in ILP mutants.

### Impaired Immature Development and Adult Reproduction of Two ILP Deletion Mutants

Dysregulation in IIS component expression might lead to altered immature development of *M. vitrata*. To test this hypothesis, larval periods were measured for three treatment groups (control,  $\Delta$ ILP1, and  $\Delta$ ILP2) to estimate developmental rate (Figure 5A). The two deletion mutants developed to pupae significantly ( $P < 0.05$ ) slowly compared with the control. In addition, developed pupae of mutants were significantly ( $P < 0.05$ ) smaller in size and weight than control pupae (Figure 5B). Adults of mutants were also significantly ( $P < 0.05$ ) smaller than control adults (Figure 5C).

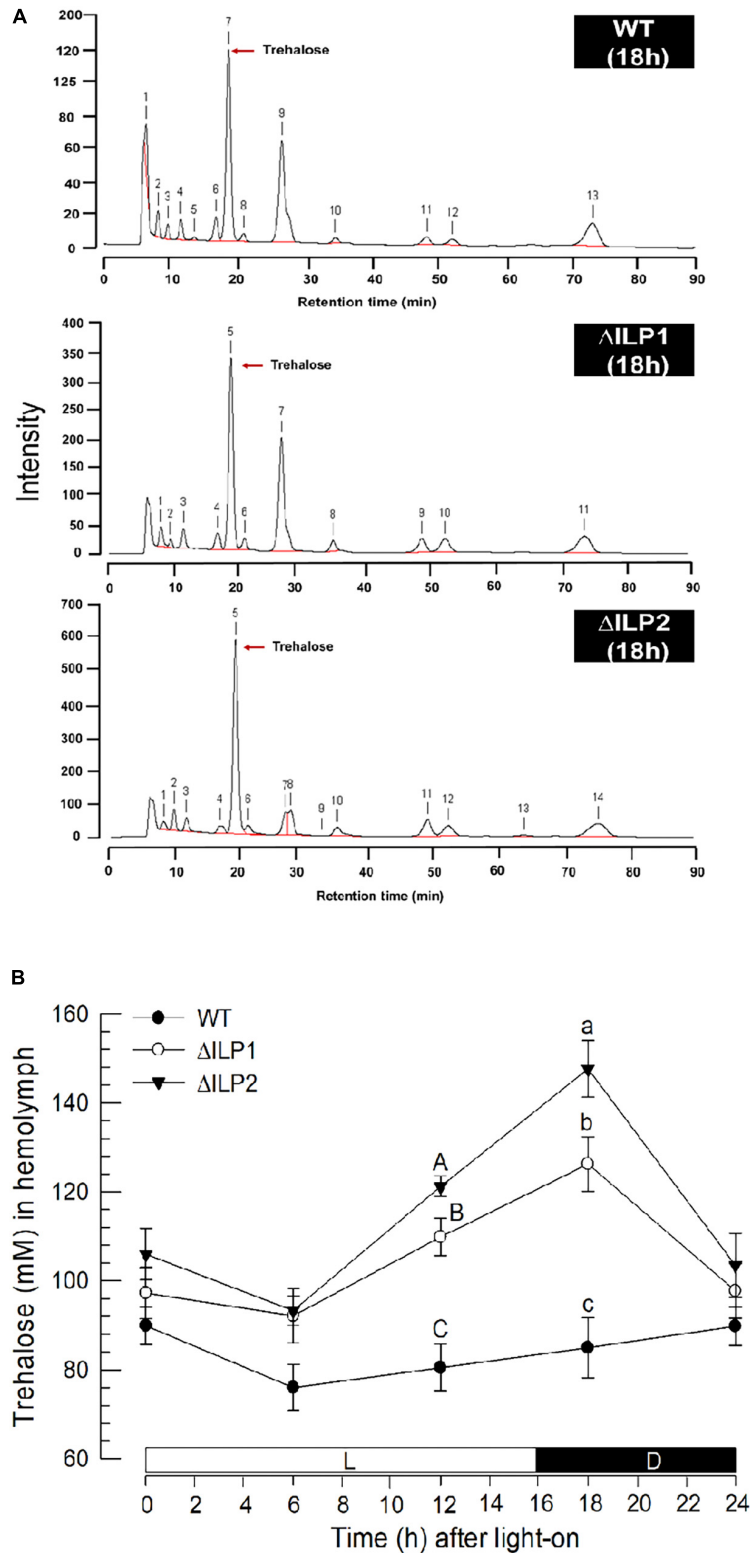
Oocyte development of *M. vitrata* underdoes three stages: previtellogenesis, vitellogenesis, and choriogenesis (Al Baki et al., 2019b). Compared with control (“WT”) females, both



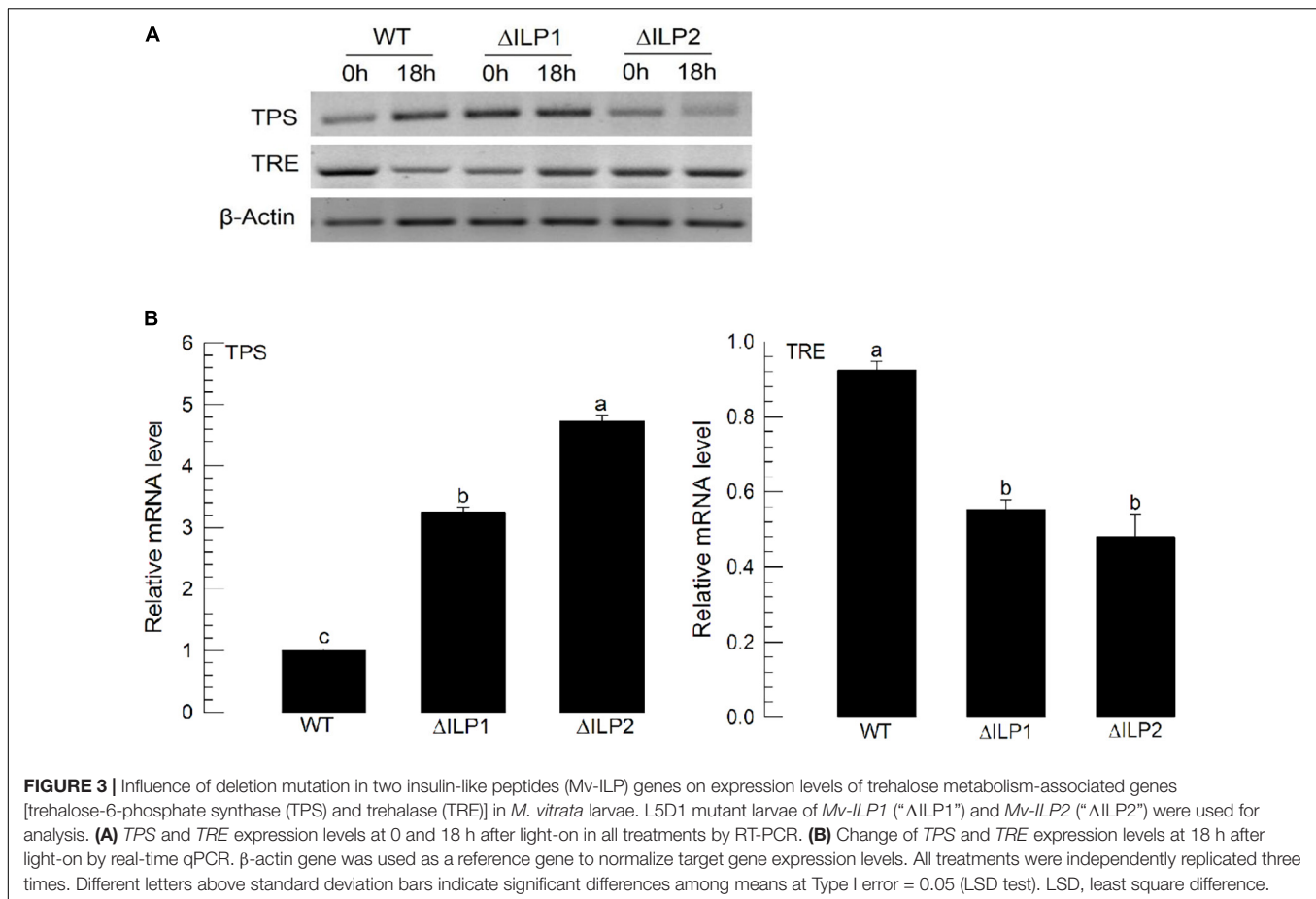
mutants ( $\Delta$ ILP1 and  $\Delta$ ILP2) showed poorly developed ovarioles in vitellogenic and chorionated oocytes (Figure 6). However, an injection of vertebrate insulin (3  $\mu$ g/insect) significantly ( $P < 0.05$ ) rescued the oocyte development.

To monitor the multiplication of oocytes at the germarium of ovarioles, DNA replication during cell division was stained

through BrdU incorporation (Supplementary Figure 1). Control females had significantly higher numbers of BrdU-labeled cells with higher intensities than the two mutants. To analyze the difference in vitellogenesis, vitellogenin (*Mv-Vg*) expression in the abdomen of females was assessed by RT-qPCR (Figure 7). Both mutants showed significantly



**FIGURE 2 |** Influence of deletion mutation of two insulin-like peptides (Mv-ILP) genes on hemolymph trehalose titers in *M. vitrata* larvae. Hemolymph was collected by cutting the first abdominal proleg of mutant L5 larvae at 18 h after light-on (L:D = 16:8 h). **(A)** Representative chromatograms of hemolymph samples were collected from control and two mutants: *Mv-ILP1* (“ΔILP1”) and *Mv-ILP2* (“ΔILP2”). **(B)** Trehalose titers in hemolymph at different time points after dsRNA injection. All treatments were independently replicated three times. Different letters above standard deviation bars indicate significant differences among means at each time point with Type I error = 0.05 (LSD test). LSD, least square difference.



( $P < 0.05$ ) decreased *Mv-Vg* expression levels compared with control females.

## Influence of ILP Mutation on Male Reproduction

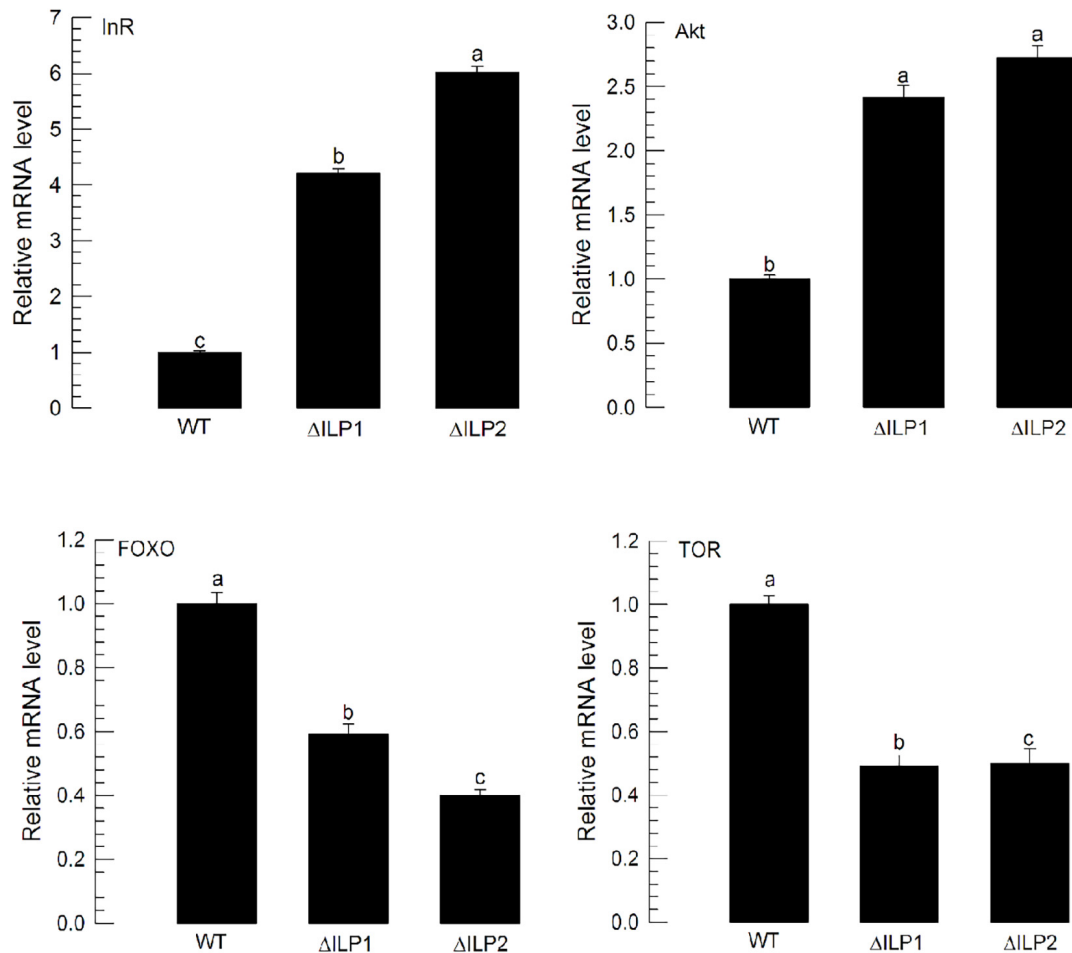
To assess male reproduction of CRISPR mutants, these mutants were then allowed to mate with wild-type individuals by reciprocal crosses (Figure 8). As expected, when mutant females mated with wild-type males, they exhibited poor reproductive activities. Interestingly, when wild-type females mated with  $\Delta$ ILP1 or  $\Delta$ ILP2 males, they (“W  $\times$  1” or “W  $\times$  2”) showed significant ( $P < 0.05$ ) decreases for the number of laid eggs (Figure 8A) and subsequent hatch rate (Figure 8B).

## DISCUSSION

Insects encode multiple ILPs that mediate various insect physiological processes (Wu and Brown, 2006). In *M. vitrata*, two ILPs have been identified (Al Baki et al., 2018a, 2020). They play important roles in mediating immature growth and adult reproduction (Al Baki et al., 2018a, 2020). Furthermore, their expression patterns are similar among different developmental stages and tissues (Al Baki et al., 2019a). Furthermore, IIS components responding to ILPs are well conserved in *M. vitrata*

(Al Baki et al., 2020). Previous studies using the RNAi approach have shown that these two ILPs mediate similar physiological processes without discriminating their independent roles. Thus, we hypothesized that these two ILPs are required to perform their mediation roles in the physiological processes of *M. vitrata*. To clarify whether they might have independent roles during entire developmental stages, we constructed two mutant lines deleting each of these two ILPs using the CRISPR/Cas9 technique.

CRISPR/Cas9 generated ILP mutants showed deletions of several nucleotides in their ORFs, leading to altered amino acid sequences or early chain termination in the two *ILP* loci of *M. vitrata*. CRISPR/Cas9 mutagenesis uses a prokaryotic adaptive machinery that enables bacteria and archaeal species to resist invading viruses and phages or plasmids (Doudna and Charpentier, 2014). The CRISPR/Cas9 system is an invaluable system for precise gene editing of diverse species, including insects, because it requires less time and effort than other genome editing techniques such as zinc finger nucleases and transcription activator-like effector nucleases (Sun et al., 2017). The current study used non-homologous end joining (NHEJ) mutagenesis of CRISPR/Cas9, in which sgRNA designed based on *Mv-ILP1* or *Mv-ILP2* sequences could specifically bind to target locus. Cas9 then could cut a specific site of the target locus. The resulting cleaved ends were then joined in a manner of NHEJ



**FIGURE 4 |** Influence of deletion mutation in two insulin-like peptides (*Mv-ILP*) genes on expression levels of four IIS component genes (*InR*, *Akt*, *FOXO*, and *TOR*) in *M. vitrata* larvae. These four gene expression levels in L5D1 larvae of mutants were quantified compared with those in the WT at 18 h after light-on.  $\beta$ -actin gene was used as a reference gene for RT-qPCR to validate cDNA integrity. Each treatment was replicated three times. Different letters above standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test). LSD, least square difference.

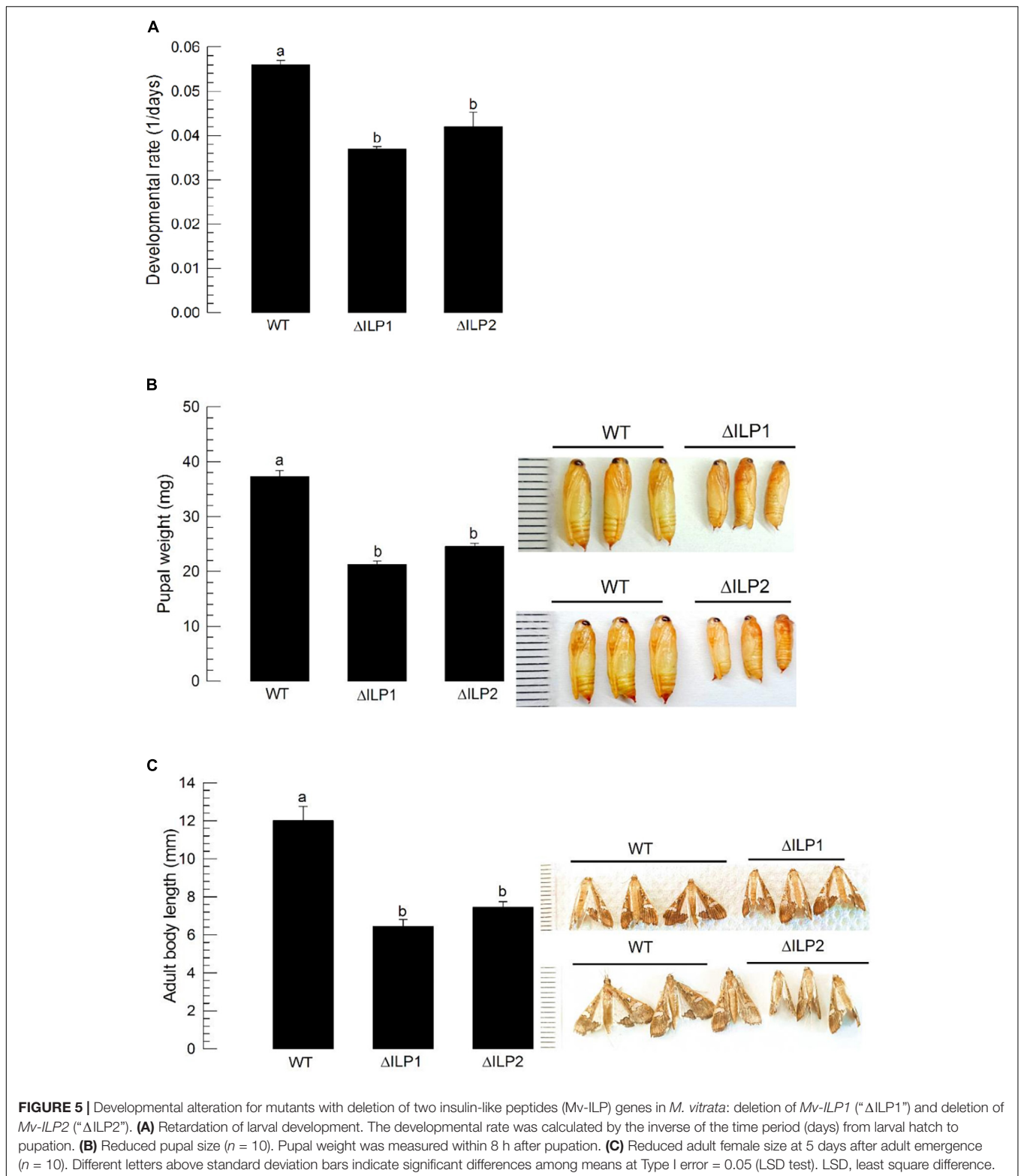
by endogenous DNA ligase activity. This NHEJ resulted in the deletion of 2–16 nucleotides for *Mv-ILP1* or 2–17 nucleotides for *Mv-ILP2*, leading to missense or nonsense mutations.

These two deletion mutants lost hemolymph sugar levels of *M. vitrata* and led to a hypertrehalosemic state. A disaccharide, trehalose, is the main hemolymph sugar of *M. vitrata* larvae, in which its titers fluctuate with feeding activity due to diel rhythmicity of feeding behavior (Al Baki et al., 2018a). Especially, feeding, including nutrient uptake, can stimulate *Mv-ILP* expression and lead to a basal level of trehalose titer, while starvation can suppress *Mv-ILP* expression and lead to a hypertrehalosemic state (Al Baki et al., 2019a). Trehalose homeostasis is mainly controlled by enzyme activities of TPS for trehalose synthesis and TRE for trehalose degradation (Becker et al., 1996). Silencing *TPS* can significantly downregulate hemolymph trehalose level, while silencing *TRE* expression can upregulate its level in *M. vitrata* (Al Baki et al., 2019a). In our current study, *ILP* mutants showed upregulated *TPS* expression but downregulated *TRE* expression. Thus, the hypertrehalosemic

state of these mutants might be due to the lack of *ILP*'s mediation effect on trehalose titer in the hemolymph of *M. vitrata*.

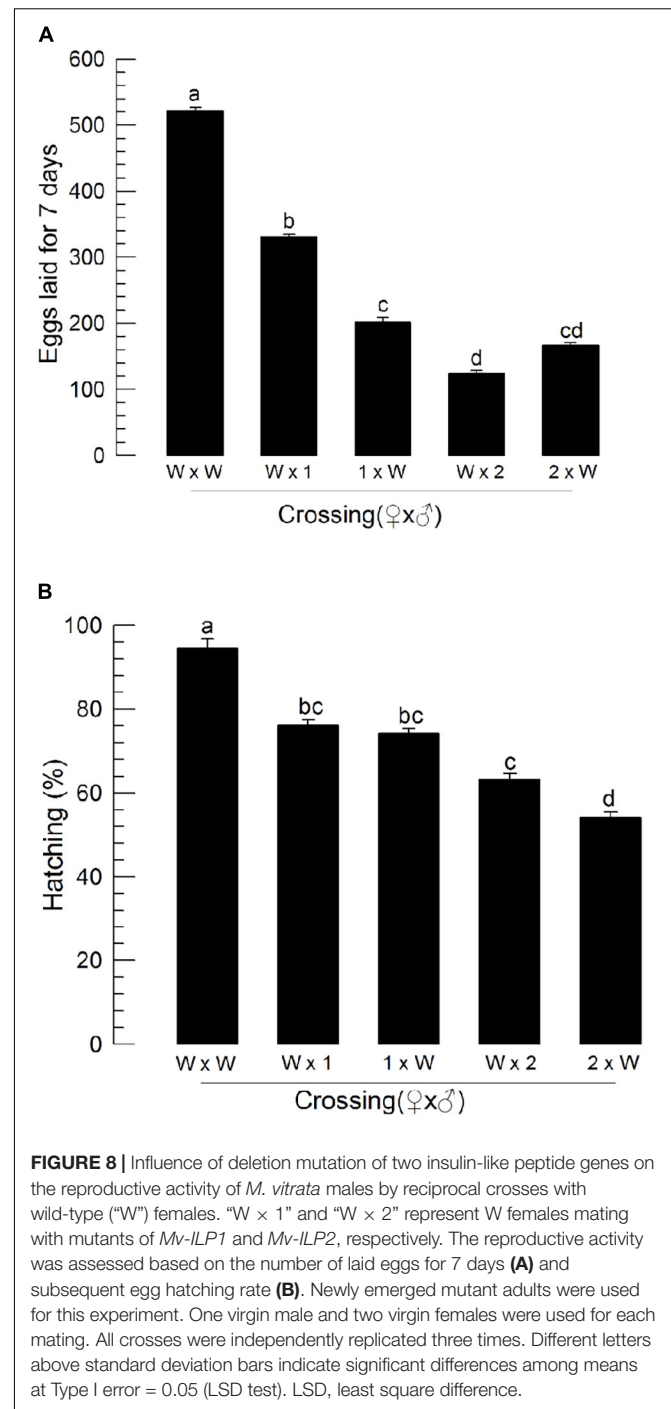
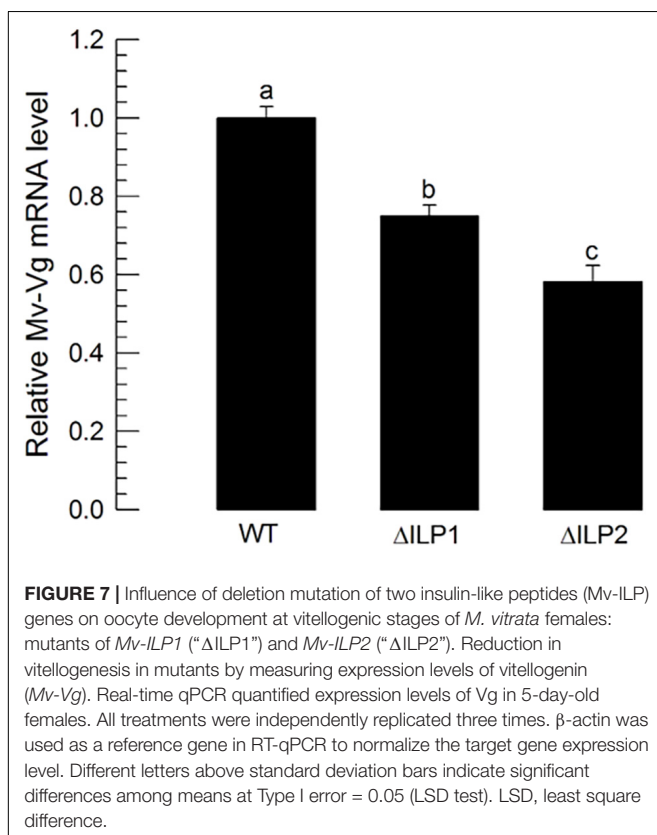
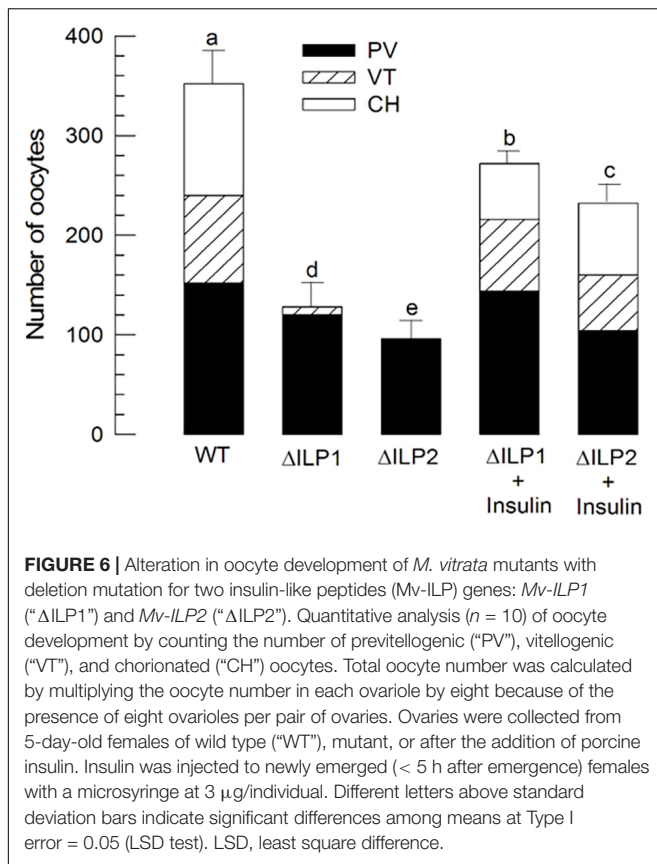
Two deletion mutants showed altered expression profiles of four IIS components and impaired immature development of *M. vitrata*. A previous study has identified seven IIS components in *M. vitrata*, including *Mv-InR*, *Mv-IRS*, *Mv-PI3K*, *Mv-PTEN* (phosphatase and tensin homolog), *Mv-FOXO*, *Mv-Akt*, and *Mv-TOR* (Al Baki et al., 2019b). Among these components, four components (*Mv-InR*, *Mv-Akt*, *Mv-FOXO*, and *Mv-TOR*) have been experimentally assessed. They are known to mediate hemolymph sugar level and immature development (Al Baki et al., 2018a, 2020). *InR* has been regarded as the sole *ILP* receptor for most insects (Wu and Brown, 2006; Wen et al., 2010). Although its expression fluctuates during immature development (Iga and Smagghe, 2011), its RNAi usually alters immature development. For example, expression levels of two *InRs* are increased during nymph-adult transition in *Aphis (Toxoptera) vitricidus* (brown citrus aphid), and their RNAi treatments result in a variety of malformed adult phenotypes along with significant





nymphal mortality (Ding et al., 2017). In *Drosophila*, FOXO activation under little ILP signal can increase *InR* expression (Puig and Tjian, 2005). Thus, the increase of *Mv-InR* expression in ILP mutants of *M. vitrata* might be explained by the lack of ILPs

and the increase of *Mv-FOXO* expression. Deletion mutants of *M. vitrata* showed increased expression of *Mv-Akt*. In *Drosophila*, Akt is activated by PDK or TOR to phosphorylate S6 ribosomal kinase (S6K), stimulating protein translation for cell growth



(Sarbasov et al., 2005). Thus, in deletion mutants of *ILP*, Akt might not be activated because of the lack of ILP and the low expression level of *Mv-TOR*. Although FOXO is a transcriptional activator in the nucleus, its phosphorylation by Akt prevents its entry into the nucleus (Mattila et al., 2009). FOXO regulates body size by regulating cell numbers in *Drosophila* (Puig et al., 2003). Thus, FOXO RNAi can promote immature development in insects. Indeed, RNAi of FOXO expression in *Gryllus bimaculatus* (cricket) can increase its body size (Dabour et al., 2011). FOXO

also regulates immature developmental rate in *M. vitrata* (Al Baki et al., 2018b). RNAi of *Mv-FOXO* expression led to developmental retardation. This is recapitulated in our current study, in which deletion mutants of *M. vitrata* exhibited a significant reduction in *Mv-FOXO* expression, leading to retardation of immature development. TOR pathway is activated by nutrient-sensing through amino acid transporters. It activates S6K and 4E-BP to initiate protein translation (Colombani et al., 2003). In *M. vitrata*, the TOR pathway is required for promoting larval development and adult reproduction (Al Baki et al., 2018a, 2019b). Especially, RNAi of *Mv-TOR* expression adversely influenced the larval developmental rate in *M. vitrata* (Al Baki et al., 2018b). Thus, the reduction of *Mv-TOR* expression in CRISPR mutants of *M. vitrata* explains the impairment of larval development.

Two ILP deletion mutants of *M. vitrata* showed impaired adult reproduction. The two ILPs of *M. vitrata* can mediate ovarian development of adult females of *M. vitrata* (Al Baki et al., 2019a). Oocyte development in the ovariole begins with active mitotic activity in the germarium, followed by vitellogenesis incorporating Vg into growing oocytes and finalized chorion formation outside mature oocytes. IIS plays a crucial role in mediating the oogenesis of *M. vitrata*, especially in previtellogenic development and Vg synthesis (Al Baki et al., 2020). Our current study showed that ILP mutants prevented BrdU incorporation in the germarium, suggesting decreased oocyte production from stem cells. In *Drosophila*, IIS regulates germline stem cell proliferation (Drummond-Barbosa and Spradling, 2001; Shim et al., 2013). Ovarian growth is arrested at the previtellogenic stage in mutants of *Drosophila* lacking IIS components (Tatar et al., 2001). The decrease of Vg expression in ILP mutants of *M. vitrata* suggests that ILP plays a crucial role in mediating Vg expression as a nutritional sensor. In *Drosophila*, the fat body can sense amino acids and send a nutritional signal called a fat body-derived signal (Géminard et al., 2009). In response to such fat body-derived signals, insulin-producing cells in the brain can produce ILPs to directly or indirectly activate Vg production (Hansen et al., 2014). Thus, the lack of nutrient signal in these deletion mutants might have prevented Vg expression. This finding was supported by the rescue of Vg expression in ILP mutants of *M. vitrata* after adding porcine insulin.

Males of ILP mutants are likely to show impaired reproductive activity. When wild-type control females mated with the mutant males, they showed a significant reduction in fecundity, and their eggs suffered low hatch rates, suggesting that mutant males of *Mv-ILP1* or *Mv-ILP2* were impaired in mating behavior or sperm fertility. In *Drosophila*, spermatogenesis begins with cell division of germline stem cells in the testis under ILP mediation because IIS mutant males show decreased numbers of germline cells, in which ILP promotes cell cycle in the

G2/M phase (Ueishi et al., 2009). These findings suggest that males of *Mv-ILP1* and *Mv-ILP2* mutants show poor spermatogenesis. This needs to be further examined in a subsequent study.

In summary, two mutant lines lacking *Mv-ILP1* or *Mv-ILP2* showed altered hemolymph sugar levels and immature development. They also showed poor reproductive activities. These suggest their functional overlap by sharing a common IIS. However, any single ILP was not enough to mediate the physiological processes. This emphasizes the requirement of both ILPs in *M. vitrata*, probably by performing their mediation cooperatively or synergistically. This interaction between two ILPs needs to be clarified in a future study.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

YK conceived and designed the study. MA and JJ performed the experiments and analyzed the data. MA drafted the manuscript. MA, JJ, and YK refined and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2021.701616/full#supplementary-material>

**Supplementary Figure 1** | Influence of deletion mutation of two insulin-like peptides (*Mv-ILP*) genes on reduction in the number of previtellogenic oocytes of *M. vitrata* females: mutants of *Mv-ILP1* ("ΔILP1") and *Mv-ILP2* ("ΔILP2"). Ovaries from 5-day-old mutant females were collected, and their ovarioles were separated. Newly dividing cells were specifically recognized by BrdU incorporation (green), while their nuclei were stained with DAPI (blue).

**Supplementary Table 1** | Primers used in this study for expression analysis.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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