© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb207019. doi:10.1242/jeb.207019

RESEARCH ARTICLE

Overexpression of PGE_2 synthase by *in vivo* transient expression enhances immunocompetency along with fitness cost in a lepidopteran insect

Shabbir Ahmed, Ariful Hasan and Yonggyun Kim*

ABSTRACT

Prostaglandins (PGs) mediate various physiological functions in insects. Specifically, PGE_2 is known to mediate immunity and egg-laying behavior in the beet armyworm, *Spodoptera exigua*. A PGE_2 synthase 2 (Se-PGES2) has been identified to catalyze the final step to produce PGE_2 in *S. exigua*. Its expression is inducible in response to immune challenge. Inhibition of the gene expression results in immunosuppression. In contrast, any physiological alteration induced by its uncontrolled overexpression was not recognized in insects. This study used the *in vivo* transient expression (IVTE) technique to induce overexpression and assessed subsequent physiological alteration in *S. exigua*. *Se-PGES2* was cloned into a eukaryotic expression vector and transfected to Sf9 cells to monitor its heterologous expression. The Sf9 cells expressed the recombinant Se-PGES2 (rSe-PGES2) at an expected size (~47 kDa), which was localized in the cytoplasm. The recombinant expression vector was then used to transfect larvae of *S. exigua*. Hemocytes collected from the larvae treated with IVTE expressed the *rSe-PGES2* gene for at least 48 h. The larvae treated with IVTE exhibited an enhanced competency in cellular immune response measured by hemocyte nodule formation. In addition, IVTE treatment of *Se-PGES2* induced gene expression of antimicrobial peptides without any immune challenge. The larvae treated with IVTE became significantly resistant to infection of an entomopathogenic nematode, *Steinernema monticolum*, or to infection to its symbiotic bacterium, *Xenorhabdus hominickii*. However, IVTE-treated *S. exigua* larvae suffered from reduced pupal size and fecundity.

KEY WORDS: Prostaglandin, PGE_2, PGES2, Immunity, Fitness, *Spodoptera exigua*

INTRODUCTION

Prostaglandins (PGs) along with other eicosanoids are oxygenated metabolites of C20 polyunsaturated fatty acids (PUFAs) derived from cellular phospholipids (PLs) by catalytic activity of phospholipase A_2 (PLA_2) (Dennis et al., 2011). Arachidonic acid (AA) is one of the PUFAs and is oxygenated by cyclooxygenase (COX) into PGs (Stanley, 2006). PGs act as dynamic lipid messengers mediating numerous homeostatic biological functions in mammals (Funk, 2001). In insects, they also act as autocrine or paracrine mediators in various physiological processes such as reproduction, secretion and immune responses (Stanley and Kim, 2014).

Among various PGs, prostaglandin E_2 (PGE_2) acts as immune mediator and performs other physiological activities such as smooth muscle dilation and contraction (Smith et al., 1991), body temperature regulation (Milton and Wendlandt, 1971) and the sleep–wake cycle in mammals (Hayaishi, 1991). In invertebrates, PGE_2 signaling mediates immune responses and influences several aspects of oogenesis (Park et al., 2005; Spracklen et al., 2014). In insects, PGE_2 stimulates egg-laying behavior of the cricket *Acheta domesticus* and the moth *Spodoptera exigua* (Stanley-Samuelson et al., 1986; Ahmed et al., 2018). Egg development in the bug *Rhodnius prolixus* is mediated by PGE_2 (Medeiros et al., 2009). In *Drosophila melanogaster*, PGE_2 mediates oocyte development by stimulating nurse cell-dumping at late oogenesis and coordinating expression of various chorion genes (Tootle et al., 2011).

PGE_2 synthase (PGES) catalyzes isomerization of prostaglandin H_2 (PGH_2) to PGE_2. Recently, we identified a membrane-bound PGES2 in *S. exigua* (*Se-PGES2*), and suppression of its expression led to physiological alterations in immunity and reproduction (Ahmed et al., 2018). In mammals, uncontrolled and excessive production of PGs leads to serious abnormalities of physiological processes (Umamaheswaran et al., 2018). In insects, immune defense consists of energy-consuming processes and requires resource allocation via competition for one or more limiting resources, leading to trade-offs between reproductive and immune processes (Schwenke et al., 2016). This led us to pose a hypothesis that uncontrolled production of PGE_2 may result in serious fitness cost in insects.

This study analyzed the change of immune responses by excessive PGE_2 production induced by overexpression of *Se-PGES2* in *S. exigua*. To overexpress *Se-PGES2*, *in vivo* transient expression (IVTE) was applied. IVTE technology has been approved to express a foreign gene in *S. exigua* (Hepat and Kim, 2012). After confirmation of the overexpression of *Se-PGES2*, changes in both cellular and humoral responses were monitored. In addition, the effect of the overexpression of *Se-PGES2* was analyzed in immature development and adult reproduction in *S. exigua*.

MATERIALS AND METHODS

Insect rearing

Larvae of *Spodoptera exigua* (Hübner 1808) were reared on an artificial diet (Goh et al., 1990) at 25±1°C. Under the rearing conditions, larvae underwent five instars (L1–L5). Adults were provided with 10% sucrose solution.

Nematode and bacterial culture

Infective juveniles (IJs) of an entomopathogenic nematode, *Steinernema monticolum*, were multiplied and harvested

Department of Plant Medicals, College of Life Sciences, Andong National University, Andong 36729, Korea.

*Author for correspondence (hosanna@anu.ac.kr)

S.A., 0000-0001-8428-0895; A.H., 0000-0002-8117-5974; Y.K., 0000-0002-6840-2167

Received 13 May 2019; Accepted 30 June 2019

according to the method described by Park et al. (1998). *Xenorhabdus hominickii* ANU101 was isolated from the nematode in a previous study (Park et al., 2017) and cultured in tryptic soy broth (TSB; BD Korea, Seoul, Korea) at 28°C for 24 h with shaking at 150 rpm (Sadekuzzaman et al., 2017). *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) was cultured in Luria–Bertani (LB) medium (BD, Franklin Lakes, NJ, USA) overnight at 37°C with shaking at 180 rpm. Heat-killed bacteria were prepared by treatment at 95°C for 10 min.

Chemicals

Arachidonic acid (AA; 5,8,11,14-eicosatetraenoic acid) and prostaglandin E_2 [PGE_2: (5Z,11α,13E,15S)-11,15-dihydroxy-9-oxoprosta-5,13-dienoic acid] were purchased from Sigma-Aldrich (Seoul, Korea) and dissolved in dimethyl sulfoxide. Anticoagulant buffer (ACB) was prepared with 186 mmol l^{-1} NaCl, 17 mmol l^{-1} Na_2EDTA and 41 mmol l^{-1} citric acid, and then adjusted to pH 4.5 with HCl.

Heterologous expression of *Se-PGES2* in Sf9 cells

A full open reading frame (ORF; 1167 bp) of *Se-PGES2* was inserted to a eukaryotic expression vector pIB/V5-His (Invitrogen) by TA cloning. After confirming the insert orientation and DNA sequence, the recombinant vector was transiently expressed in the Sf9 cell line by cationic lipid-mediated transfection using X-treme GENE 9 DNA transfection reagent (Roche, Mannheim, Germany). Sf9 cells (~5×10^5 cells ml^{-1}) were seeded into a 75 cm^2 tissue culture flask (Nunc, Jiangsu, China) in TC100 insect culture medium (Welgene, Daegu, Korea) containing 5% fetal bovine serum. Following the manufacturer's protocol, recombinant vector DNA (3 µg) was mixed with 15 µl of transfection reagent and incubated for 30 min at 25°C. After 30 min of incubation, the DNA lipid complex was added into the flask containing Sf9 cells. After 24 h of cell culture, the medium was replenished with 15 ml of fresh medium without serum. Cells were further incubated for 72 h at 28°C. To extract the proteins from the cultured cells, Sf9 cells were centrifuged at 3000 ***g*** for 5 min at 4°C. The pellet was washed with 5 ml of 100 mmol l^{-1} phosphate-buffered saline (PBS; pH 7.4) and lysed with a lysis buffer containing 50 mmol l^{-1} Tris HCl, 150 mmol l^{-1} NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol l^{-1} EDTA and protease inhibitor cocktail (Spruce, St Louis, MO, USA). After 5 min lysis on ice with intermittent mixing, cells were centrifuged at 12,500 ***g*** for 5 min. Supernatant containing proteins was quantified using the Bradford (1976) method.

In vivo transient expression (IVTE)

A recombinant vector described above was mixed with a transfection reagent Metafectene PRO (Biontex, Plannegg, Germany) in 1:1 (v/v) ratio and then incubated at 25°C for 30 min to form liposomes to increase transfection efficiency. One microgram of vector was injected into 1-day-old L5 larvae (L5D1) using a microsyringe (Hamilton, Reno, NV, USA) equipped with a 26 gauge needle. To extract the proteins from the larvae, total hemolymph (~500 µl) from ten L5 individuals was collected from larval proleg, mixed with 500 µl of ACB. Hemolymph was then centrifuged at 3000 ***g*** for 5 min at 4°C. Pellet was washed with 5 ml of PBS and lysed with a lysis buffer as described above. After 5 min lysis on ice with intermittent mixing, cells were centrifuged at 12,500 ***g*** for 5 min to obtain supernatant proteins.

Western blotting

Extracted proteins (~100 µg $sample^{-1}$) were separated on 10% SDS-PAGE. The separated proteins in the gel were transferred onto a 0.2 µm pore nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) for 45 min at 100 V in a chilled transfer buffer (25 mmol l^{-1} Tris, 190 mmol l^{-1} glycine and 20% methanol, pH 8.5). The membrane was briefly rinsed in Tris-buffered saline with Tween 20 (TBST) (20 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl and 0.1% Tween 20, pH 7.5) and then blocked for 1 h at room temperature (RT) in TBST containing 3% bovine serum albumin (BSA). Membranes were then incubated for 2 h at 4°C in Anti-V5 antibody (Invitrogen) as a primary antibody at a dilution of 1:5000 with TBST containing 3% BSA. Membranes were then washed three times in TBST for 5 min per washing and then incubated with anti-mouse IgG-alkaline phosphatase secondary antibody (Sigma-Aldrich, Korea) at a dilution of 1:30,000 in TBST containing 3% BSA for 1 h at RT. Blot was rinsed three times with TBST. To detect alkaline phosphatase activity, nitrocellulose membrane was incubated with a substrate (BICP/NBT, Sigma-Aldrich).

Immunofluorescence assay (IFA)

One microliter of recombinant pIB vector containing *Se-PGES2* was injected into L5D1 larvae at a dose of 1 µg $larva^{-1}$. After 24 h post-injection (hpi), IFA was performed. Briefly, total hemolymph (~150 µl) from five L5 individuals was collected from larval proleg, mixed with 850 µl of ACB and incubated on ice for 30 min. After centrifugation at 180 ***g*** for 2 min at 4°C, 700 µl of supernatant was discarded. The cell suspension was gently mixed with 700 µl of TC100 insect tissue culture medium (Welgene). Ten microliters of this hemocyte suspension was placed onto a glass coverslip and incubated in a wet chamber under darkness. Cells were then fixed with 4% paraformaldehyde for 10 min at RT. After washing three times with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min at RT. Cells were washed once in PBS and blocked with 5% skim milk in PBS for 10 min at RT. After washing once with PBS, cells were incubated with 5% of Alexa Fluor 555 phalloidin (Invitrogen) and 0.2% fluorescein isothiocyanate (FITC)-tagged Anti-V5 antibody (Invitrogen) individually in PBS for 1 h at RT. After washing three times, cells were incubated with 0.1% of 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific Korea) in PBS for 5 min. Finally, after washing twice in PBS, cells were observed under a fluorescence microscope (DM2500, Leica, Wetzlar, Germany) at 400× magnification.

In Sf9 cells, cells were collected at 24 h after transfection as described above and centrifuged at 180 ***g*** for 2 min at 4°C. Pellet cells were gently mixed with 500 µl of TC100 insect tissue culture medium (Welgene) and the IFA procedure was followed described above.

RNA extraction and RT-qPCR

Total RNA was extracted from hemocytes of ten L5 larvae of *S. exigua* as an experimental unit. By cutting a proleg, hemolymph was collected in cold ACB and then centrifuged at 800 ***g*** for 5 min at 4°C. The resulting hemocyte pellet was used to extract total RNA with Trizol reagent (Invitrogen) according to the manufacturer's instructions. After DNase treatment, 1 µg of total RNA was used to prepare first-strand cDNA synthesized by RT-Premix oligo-dT [5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC-T(16)-3′, Intron Biotechnology, Seoul, Korea] in a reaction volume of 20 µl. The synthesized single-stranded cDNA was used as a template for PCR amplification with 35 rounds of a temperature cycle (95°C for 1 min, 52°C for 1 min and 72°C for 1 min) after an

Journal of Experimental Biology (2019) 222, jeb207019. doi:10.1242/jeb.207019

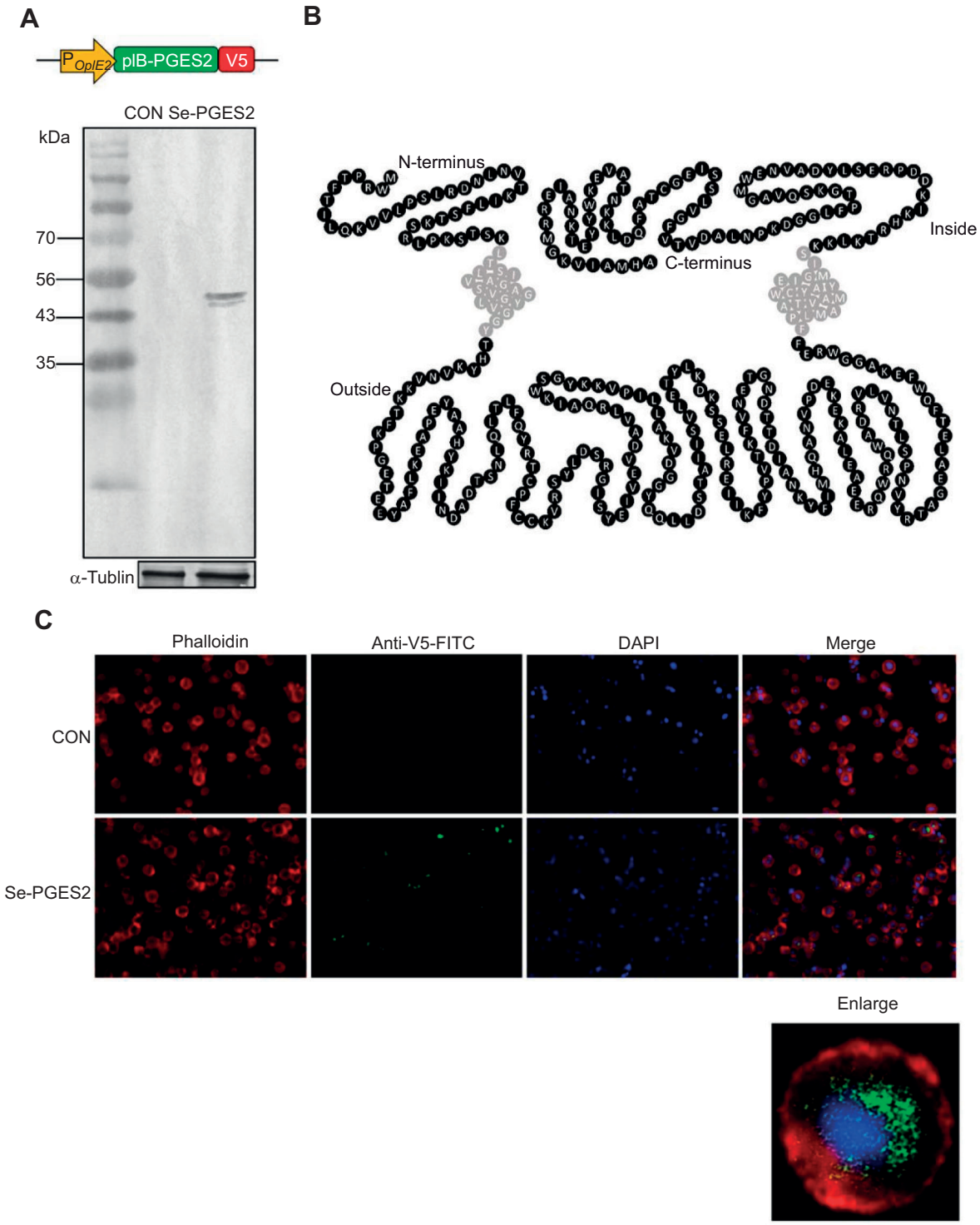

Fig. 1. Gene cloning and heterologous expression of *Se-PGES2* in *Spodoptera exigua* Sf9 cells. (A) Gene cloning and heterologous expression of *Se-PGES2* in Sf9 cells. (A) Expression vector organization and western blot analysis. Expression of *Se-PGES2* was driven under baculoviral immediate-early promoter (pIE). The recombinant protein size (rSe-PGES2) was ~47 kDa, which was recognized by V5 antibody. (B) Transmembrane domain analysis of Se-PGES2. The domains of Se-PGES2 were predicted using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). (C) Immunofluorescence assay for the detection of transient expression of rSe-PGES2 in Sf9 cells. F-actin was specifically detected with Alexa Fluor® 555 phalloidin whereas nucleus was stained with DAPI. For checking transient expression, Anti-V5-FITC antibody was used. To confirm localization of rSe-PGES2 in Sf9 cells, the cells were observed under a fluorescence microscope at 1000× magnification. CON, control.

initial heat treatment step at 95°C for 5 min with gene-specific primers (Table S1). The PCR products were separated on 1% agarose gel under 100 V and subsequently stained with ethidium bromide. RT-qPCRs were performed with a qPCR instrument (CFX Connect Real-Time PCR Detection System, Bio-Rad Laboratories) using SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) according to the general guideline suggested by Bustin et al. (2009). A ribosomal protein, RL32, gene was used as a stably

Journal of Experimental Biology (2019) 222, jeb207019. doi:10.1242/jeb.207019

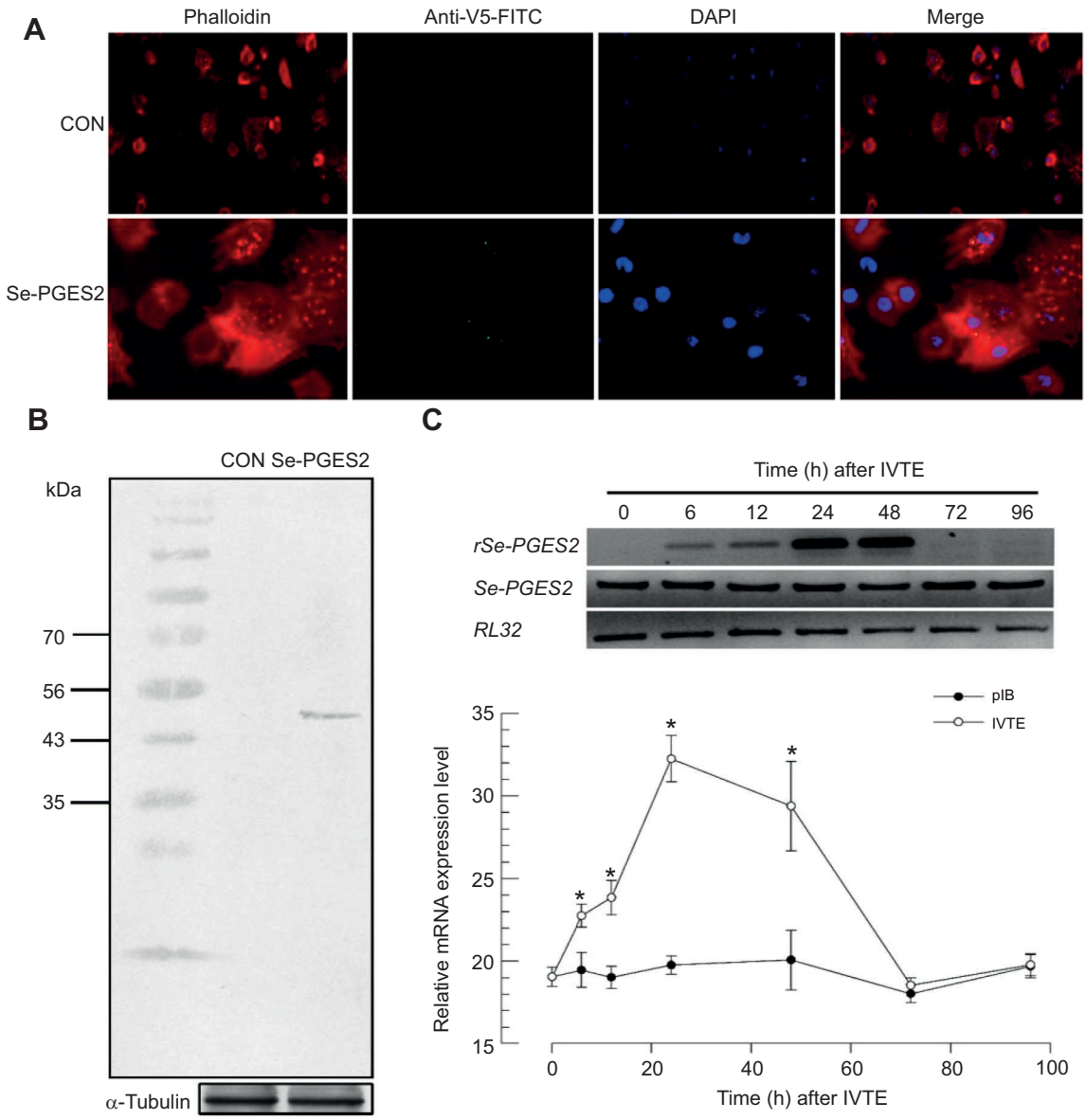

Fig. 2. *In vivo* transient expression (IVTE) of *Se-PGES2* in *S. exigua* hemocytes. (A) Immunofluorescence assay for transient expression of *Se-PGES2* in hemocytes using Anti-V5-FITC. (B) Western blot analysis of recombinant Se-PGES2 (~47 kDa) with V5 antibody. (C) Enhanced gene expression of *Se-PGES2* after IVTE. Expression levels were compared between larvae injected with empty (pIB) or recombinant (IVTE) vector. In IVTE-treated larvae, expression of recombinant Se-PGES2 (*rSe-PGES2*) gene was detected with primer specific to the V5 tag. A ribosomal gene, *RL32*, was used as reference gene. Each treatment was replicated three times. Each treatment used 10 larvae. *Significant difference among means at Type I error=0.05 (LSD test).

expressed reference gene in *S. exigua* (Park et al., 2015) for qPCR with gene-specific primers (Table S1). Each cycle was scanned by measuring fluorescence intensity to quantify the PCR products. After the PCR reactions, melting curve analyses were performed from 60 to 95°C to ensure consistency and specificity of the amplified products. Each treatment was replicated three times using independent RNA collections. Quantitative analysis of gene expression was performed using the comparative C_T ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001).

Nodulation assay

A nodulation assay was performed at 24 h after IVTE (1 µg larva^{-1}) treatment. Briefly, heat-killed *E. coli* (~4.2×10^4 cells larva^{-1}) was injected in larvae through an abdominal proleg. The treated larvae were then incubated for 8 h at 25°C. After they were kept in 4°C to prevent intentional movement, they were fixed on paraffin plate using insect pins and dissected. The melanized nodules in the hemocoel were counted on gut and fat body under a stereoscopic microscope (Stemi SV11, Zeiss, Jena, Germany) at 50× magnification. Each treatment used 10 larvae. Each treatment was independently replicated three times. All assessments were performed by a blind test.

Antimicrobial peptide (AMP) expression analysis

Recombinant vector (1 µg larva^{-1}) for IVTE or PGE_2 (1 µg larva^{-1}) was injected into the hemocoel of L5 larvae as described above. At 24 hpi for IVTE or 8 hpi for PGE_2, three tissues (hemocytes, fat body and midgut) were isolated and used for RNA extraction. For each replicate, 10 larvae were used for collecting tissues and subsequent RNA extraction. Each treatment used three independent sample collections (n=30 larvae). Eleven AMPs were assessed for their expression levels by RT-qPCR using gene-specific primers (Table S1).

Journal of Experimental Biology (2019) 222, jeb207019. doi:10.1242/jeb.207019

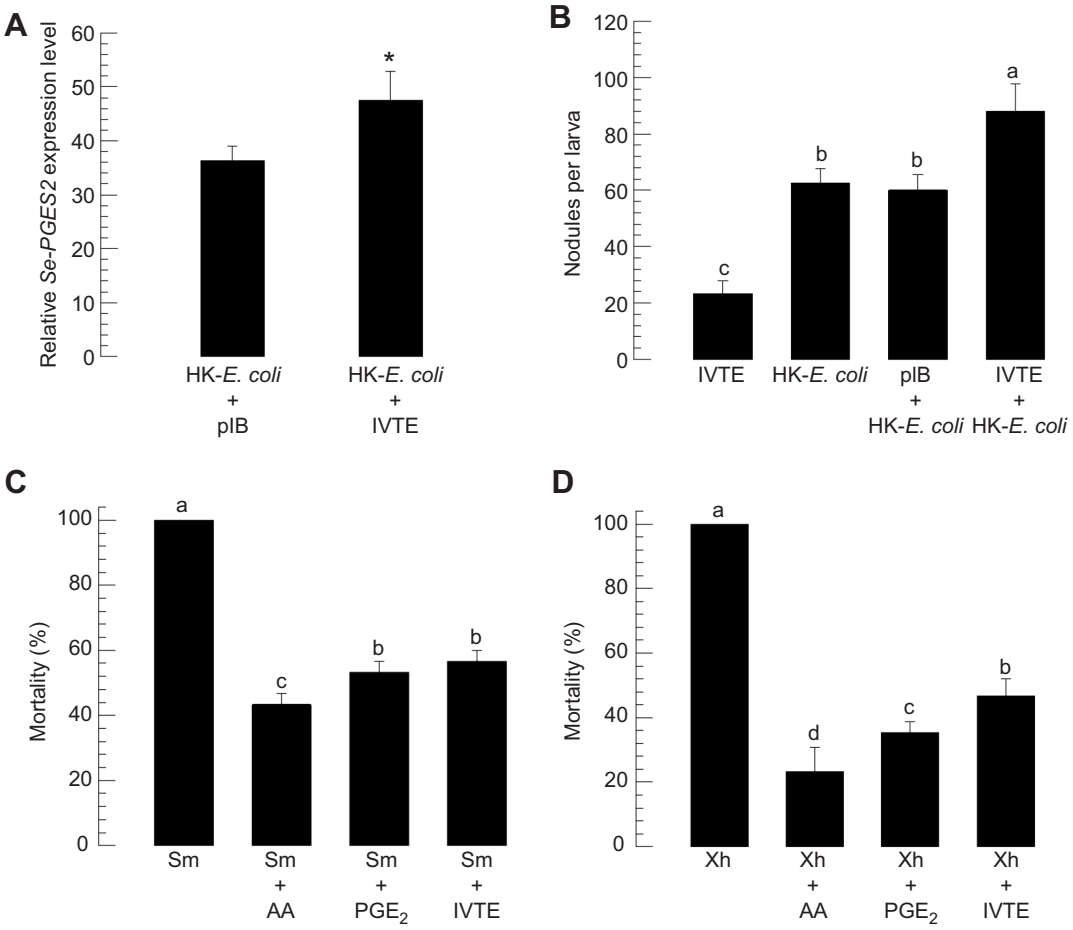

Fig. 3. IVTE of *Se-PGES2* leads to immunocompetence and enhances resistance against entomopathogen infection. (A) Enhanced gene induction level of *Se-PGES2* in larvae injected with recombinant vector (IVTE) compared with larvae injected with empty vector (pIB) after bacterial challenge. *Se-PGES2* expression levels were measured at 8 h after injection of heat-killed (HK) *Escherichia coli* (~4.2×10^4 cells larva^{-1}) L5 larvae. *Significant difference between treatments at Type I error=0.05 (LSD test). (B) Enhanced nodule formation in IVTE-treated larvae compared with control (pIB) larvae. (C,D) Enhanced resistance of IVTE-treated larvae against a nematode, *Steinernema monticolum* (Sm) (C) or a bacterium, *Xenorhabdus hominickii* (Xh) (D). At 24 h after IVTE, the nematode (1000 IJ larva^{-1}) or bacterial (~3.5×10^4 CFU larva^{-1}) treatment was performed. In contrast, an addition of AA (10 µg larva^{-1}) or PGE_2 (1 µg larva^{-1}) to the pathogen infection was assessed. Each treatment was replicated three times. Each replication used 10 larvae. Different letters above standard deviation bars indicate significant difference among means at Type I error=0.05 (LSD test).

Pathogenicity test

Recombinant vector (1 µg larva^{-1}) for IVTE was injected into the hemocoel of L5 larvae. At 24 hpi, nematode or bacteria was sprayed onto or injected into the larvae, respectively. For the nematode pathogenicity test, 1000 IJ larva^{-1} of *S. monticolum* were sprayed onto L5 larva in a Petri dish (5 cm diameter, 3 cm height). For the bacterial pathogenicity test, *X. hominickii* (~3.5×10^4 CFU larva^{-1}) was injected into L5 larvae. Larvae injected with empty vector were used for controls. For the rescue experiment, AA (10 µg larva^{-1}) or PGE_2 (1 µg larva^{-1}) was injected along with the nematode or bacterial treatment. Each treatment used 10 larvae and was independently replicated three times.

Developmental assay

One microgram of the recombinant vector for IVTE was injected into larvae (within an hour after emerging into L5). Larvae injected with empty vector were used for controls. Pupal mass was measured on the first day after pupation. Each treatment was replicated three times, and each replicate consisted of 10 insects. A day after adult emergence, treated males and females were kept together for mating. Total numbers of eggs laid were counted for 3 days. Each treatment used five pairs and was replicated three times.

Statistical analysis

Data from all assays were analyzed by one-way ANOVA using PROC GLM for continuous variables in SAS (SAS Institute, Cary, NC, USA). Percent data were treated by arcsine transformation. All variables were tested against a normal distribution based on the Shapiro–Wilk test using PROC UNIVARIATE. The means were compared using the least squared difference (LSD) test at a Type I error of 0.05.

RESULTS

Cellular localization of Se-PGES2

The cellular locality of Se-PGES2 was assessed by its heterologous expression in Sf9 cells transfected with a eukaryotic expression vector containing *Se-PGES2* (Fig. 1). The recombinant *Se-PGES2* (*rSe-PGES2*) was expressed in Sf9 cells and the recombinant protein was confirmed by western blot at the expected size (~47 kDa) (Fig. 1A). This protein spanned the membrane two times (Fig. 1B). LocTree 3 (https://rostlab.org/

Journal of Experimental Biology (2019) 222, jeb207019. doi:10.1242/jeb.207019

services/loctree3/) and LocSigDB (http://genome.unmc.edu/LocSigDB/) predicted cellular locality of Se-PGES2 in the endoplasmic reticulum, Golgi body, mitochondria or lysosome. IFA showed that the rSe-PGES2 protein was localized in the cytosol of Sf9 cells (Fig. 1C).

IVTE of Se-PGES2 in *S. exigua* hemocytes

After 24 hpi of pIB vector containing *Se-PGES2* in L5 larvae, its expression was analyzed in hemocytes by IFA (Fig. 2). This IVTE induced *rSe-PGES2* expression because rSe-PGES2 proteins were observed in the cytosol of *S. exigua* hemocytes (Fig. 2A). IVTE was further confirmed by western blot analysis (Fig. 2B). To monitor *rSe-PGES2* expression in L5 larvae, RT-PCR primers were designed from its V5 tag to discriminate it from endogenous *Se-PGES2* transcripts (Table S1). Total (endogenous+recombinant) *Se-PGES2* expression levels significantly ($F_{1,40}$=203.90, P<0.05) increased from 6 to 48 hpi whereas the endogenous *Se-PGES2* expression levels were not significantly different (Fig. 2C). The enhanced *Se-PGES2* expression levels by IVTE treatment were not detected after 72 hpi.

Effect of overexpressed Se-PGES2 on cellular immunity

When the IVTE-treated larvae were challenged with bacteria, the total *Se-PGES2* expression level was further elevated compared with that of control larvae (pIB) challenged with the bacteria (Fig. 3A). Under these conditions of overexpression of *Se-PGES2*, a cellular immune response was assessed by counting nodules in response to bacterial challenge (Fig. 3B). Larvae treated with IVTE of *rSe-PGES2* formed significantly ($F_{3,36}$=189.14, P<0.05) higher numbers of nodules (~88 nodules larva^{-1}) than control larvae (~62 nodules larva^{-1}). It was interesting that larvae treated with IVTE of *rSe-PGES2* formed nodules (~21 nodules larva^{-1}) without bacterial challenge.

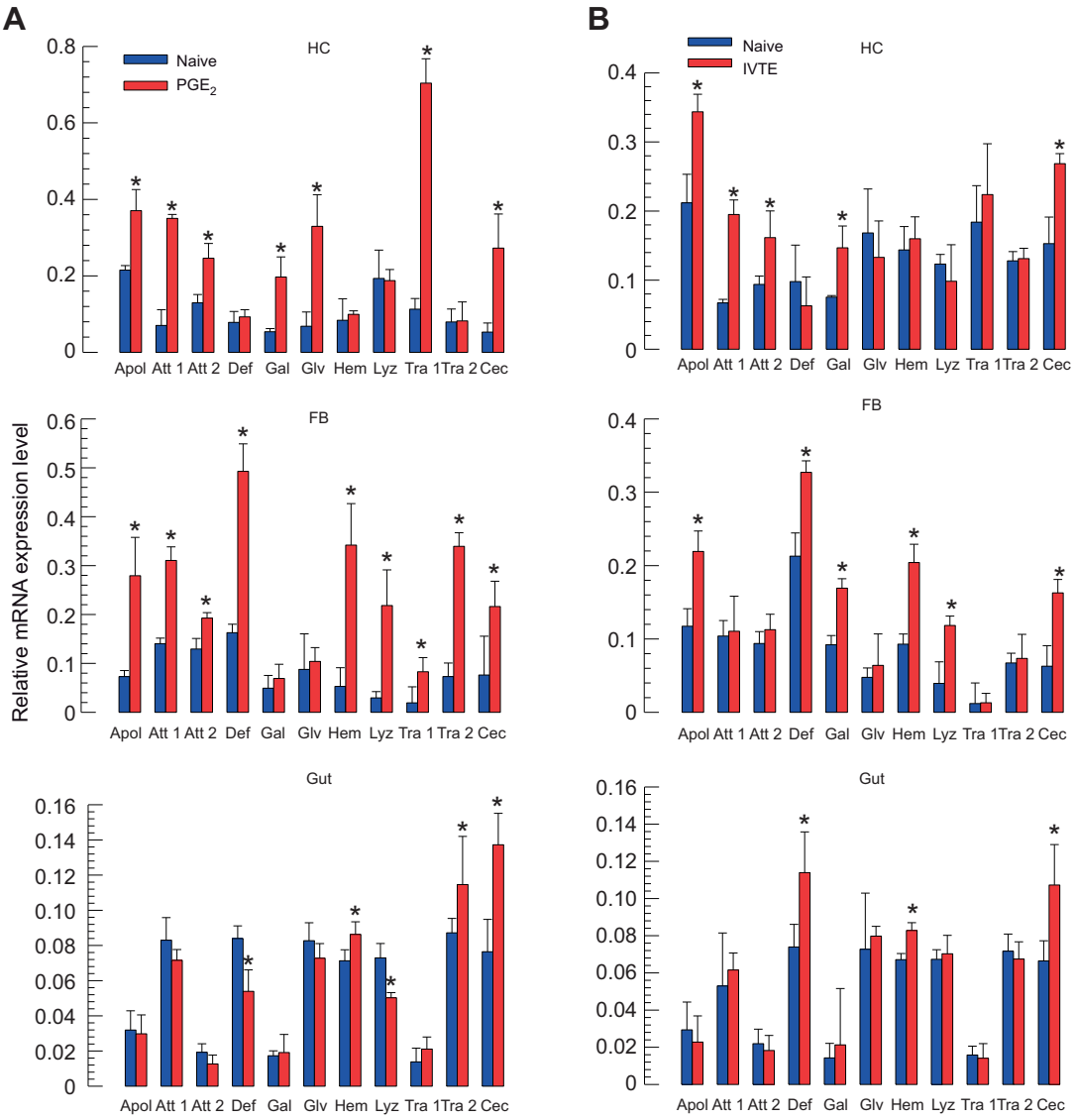

Fig. 4. Effect of PGE_2 and IVTE on AMP gene expression. (A) Effect of PGE_2 on AMP gene expression. PGE_2 (1 µg larva^{-1}) was injected into L5 larvae of *S. exigua*. At 8 hpi, hemocyte (HC), fat body (FB) and midgut (Gut) were used for expression profile analysis of 11 AMP genes: *apolipophorin III* (Apol), *attacin 1* (Att 1), *attacin 2* (Att 2), *defensin* (Def), *gallerimycin* (Gal), *gloverin* (Glv), *hemolin* (Hem), *lysozyme* (Lyz), *transferrin 1* (Tra 1), *transferrin 2* (Tra 2) and *cecropin* (Cec). (B) Effect of IVTE of *Se-PGES2* on AMP gene expression. IVTE of *Se-PGES2* (1 µg larva^{-1}) was injected into L5 larvae of *S. exigua*. At 24 hpi, hemocyte, fat body and midgut were used for expression profile analysis of 11 AMP genes. RL32 was used as an internal control. Each treatment was replicated three times. Each replication used a pooled mRNA of 10 larvae. All expression levels were relative to the highest expression value of *Tra 1* in hemocytes treated with PGE_2. *Significant difference among means at Type I error=0.05 (LSD test).

Journal of Experimental Biology (2019) 222, jeb207019. doi:10.1242/jeb.207019

Effect of overexpressed Se-PGES2 on humoral immunity

The influence of PGE_2 injection on expression levels of AMP genes of *S. exigua* was monitored without immune challenge (Fig. 4A). All 11 AMPs were inducible in their expression in response to PGE_2. However, the inducible AMP genes varied among different tissues. *Cecropin* expression was significantly ($F_{1,16}$=43.88, P<0.05) enhanced in all three tissues. Except *gallerimycin* and *gloverin*, nine other AMP genes were induced in their expression levels in fat body. Hemocytes induced seven AMP genes including *gallerimycin* and *gloverin* in response to PGE_2. Compared with hemocytes or fat body, the gut exhibited overall lower expression levels (0.01–0.16 in relative mRNA levels).

Similar inducible expression profiles of AMP genes were observed in larvae treated with IVTE of *rSE-PGES2* (Fig. 4B). *Cecropin* expression was also significantly ($F_{1,16}$=9.52, P<0.05) enhanced in all three tissues in response to IVTE treatment. Six AMP genes significantly ($F_{1,34}$=23.04, P<0.05) increased their expression levels in fat body and five of them were inducible in PGE_2 injection. Five AMP genes (*apolipophorin III*, *attacin 1*, *attacin 2*, *gallerimycin* and *cecropin*), which were inducible to PGE_2 injection, were significantly ($F_{1,28}$=18.54, P<0.05) enhanced in their expression levels in hemocytes. Three immune-associated genes (*defensin*, *hemolin* and *cecropin*) that were inducible to PGE_2 injection were also induced by IVTE treatment in gut.

Effect of overexpressed *Se-PGES2* on resistance of *S. exigua* larvae against pathogen infection

Enhanced cellular and humoral immune responses by IVTE of *rSe-PGES2* were likely to be advantageous for *S. exigua* larvae to defend against entomopathogens. To test this hypothesis, two entomopathogens of *S. monticolum* (Fig. 3C) and *X. hominickii* (Fig. 3D) were used to infect larvae of *S. exigua* treated along with IVTE. At nematode or bacterial doses to kill 100% of larvae, an addition of PGE_2 or its biosynthetic precursor (=arachidonic acid) significantly ($F_{2,27}$=595.50, P<0.05) rescued the larvae. IVTE treatment also significantly ($F_{1,18}$=596.50, P<0.05) rescued the larvae from the nematode or bacterial infection.

Trade-off of *Se-PGES2* overexpression on larval growth and adult fecundity

Without any immune challenge, overexpressed *Se-PGES2* levels resulted in nodule formation and increased AMP gene expression levels, suggesting uncontrolled immune responses. These unnecessary immune responses might lead to adverse effects on larval development and adult reproduction. Indeed, larvae treated with IVTE of *rSe-PGES2* exhibited delayed development and led to significantly reduced size of pupae (t=4.11, d.f.=9, P<0.05; Fig. 5A). Adults emerged from the pupae exhibited a significantly lower number of laid eggs (t=7.04, d.f.=9, P<0.05; Fig. 5B).

DISCUSSION

Various physiological processes are mediated by PGE_2 in insects (Stanley and Kim, 2019). Thus, biosynthesis of PGE_2 by Se-PGES2 plays a crucial role in immunity and reproduction of *S. exigua* (Ahmed et al., 2018). The results of the present study support its significance in mediating both cellular and humoral immune responses, and also suggest that a tight regulation of *Se-PGES2* expression is necessary to avoid excess immune responses.

Heterologous expression of *rSe-PGES2* in Sf9 cells and its IVTE in *S. exigua* hemocytes indicated that Se-PGES2 is localized in cytoplasm. In addition, bioinformatics analysis suggests that it is localized in the endoplasmic reticulum. To synthesize PGE_2 in mammals, PLA_2 catalyzes PLs to release AA, which is then oxygenated by COX to produce PGH_2 (Burke and Dennis, 2009; Stanley, 2006; Park et al., 2014). In mammals, cytosolic or membrane-bound PGES isomerizes PGH_2 to PGE_2. Insects also have been considered to have all biosynthetic machinery to synthesize PGE_2, as predicted in previous bioinformatics analysis of *Drosophila melanogaster* (Scarpati et al., 2019). Indeed, three different PLA_2s have been identified in *S. exigua* and include one secretory PLA_2 and two cellular PLA_2s. Also, COX-like peroxynectins have been identified and play a role in oxygenating AA (Park et al., 2014). Thus, along with identification of Se-PGES2 (Ahmed et al., 2018), a biosynthetic pathway of PGE_2 has been identified in *S. exigua*. In that study, Se-PGES2 was predicted to be a membrane-bound type among three different types of PGESs. The present study, using the heterologous expression in Sf9 cells, supports the cytosolic locality, at which Se-PGES2 performs its PGE_2 biosynthesis.

IVTE of *rSe-PGES2* upregulated total *Se-PGES2* expression levels for at least 48 hpi in *S. exigua*. Transient expression of a foreign gene has been usually performed by transfection of recombinant genes in an expression vector to cell lines via liposome formation to enhance DNA entry to target cells (Felgner et al., 1987). IVTE, which has been done by direct transfection to post-embryonic tissues, was also successfully used to deliver DNA into larval tissues of *Bombyx mori* (Xiao et al., 2002). Indeed, Hepat and Kim (2012) tested the applicability of the IVTE technique in

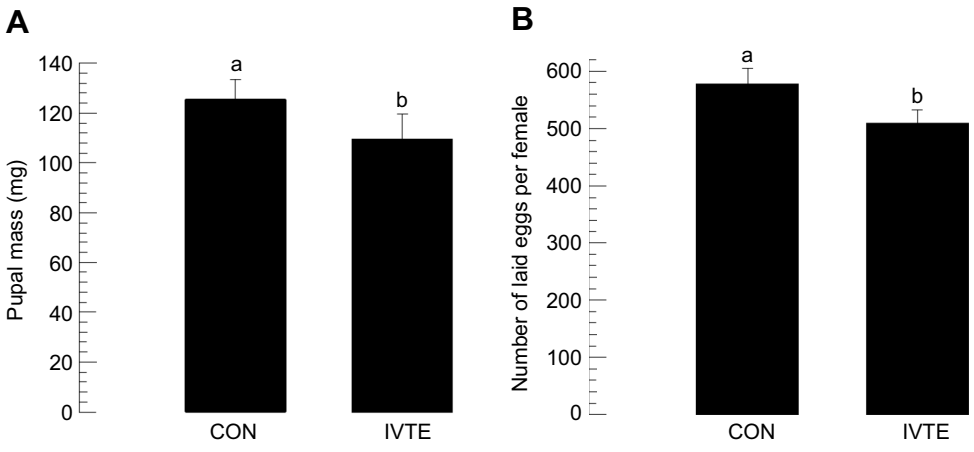

Fig. 5. Influence of overexpressed *Se-PGES2* by IVTE on larval development and adult fecundity of *S. exigua*. (A) Reduced pupal mass induced by IVTE of *Se-PGES2*. L5D1 larvae were injected with recombinant pIB vector (1 µg larva^{-1}). Soon after pupal molting, pupal mass was measured. Each treatment was replicated three times. Each treatment used 10 larvae. (B) Reduced fecundity induced by IVTE of *Se-PGES2*. After adult emergence, adult fecundity was measured by counting laid eggs per female. Each treatment used five pairs of adults. Each treatment was replicated three times. Different letters above standard deviation bars indicate significant difference among means at Type I error=0.05 (LSD test).

S. exigua using a recombinant pIB vector containing *enhanced green fluorescence protein* (*EGFP*) and showed that *EGFP* expression occurred mostly in hemocytes and fat body and persisted with the dependency of expression levels on injection dose of the vector.

Overexpression of *Se-PGES2* induced by IVTE enhanced both cellular and humoral immune responses. The enhanced immunocompetency via IVTE of *Se-PGES2* was supported by the effective defense against infection of entomopathogens. Our previous study showed that Se-PGES2 is associated with cellular immune response presumably by enhancing PGE_2 biosynthesis (Ahmed et al., 2018). The additional PGE_2 might facilitate the cellular and humoral immune responses, which would explain the enhanced survivorship against pathogen infection. Miller (2005) showed that hemocyte-spreading behavior increases in a dose-dependent manner of the amount of treated PGE_2 in *Manduca sexta* larvae. The role of PGE_2 in hemocyte spreading may be induced by activating actin remodeling by the growth of F-actin via activation of the actin-bundling protein Fascin (Groen et al., 2012). PGE_2 also increased AMP production in a dose-dependent manner in *S. exigua* (Shrestha and Kim, 2009). Toll and IMD immune signaling pathways are identified in *S. exigua* (Hwang et al., 2013). A Toll signaling component, Pelle, activates PLA_2 activity, presumably to increase PGE_2 production (Shafeeq et al., 2018). Yajima et al. (2003) showed that the PLA_2 activity is associated with IMD immune signaling to produce AMPs in *Drosophila*. Furthermore, Zhang et al. (2018) showed that PGs rescued *Spodoptera frugiperda* larvae infected with an entomopathogen, *Serratia marcescens*.

Overexpression of *Se-PGES2* induced by IVTE had an adverse effect on development and reproduction of *S. exigua*. The uncontrolled expression of Se-PGES2 may lead to untimely production of PGE_2. The excess PGE_2 may divert the nutrient usage from development and reproduction to energy-consuming immune responses even without pathogen infection, which resulted in fitness costs such as reduction in pupal body mass and adult fecundity. In the honey bee, immune challenge reduces expression and accumulation of stored plasma proteins such as vitellogenin and hexamerin, whereas it highly induces immune-associated genes, *defensin* and *prophenoloxidase*, suggesting an adaptive strategy to redirect resources to combat injury or infection (Lourenço et al., 2009). In *S. frugiperda*, in which PGs played a crucial role in mediating cellular immune response, the PG-treated larvae suffered reduced survivorship compared with controls (Zhang et al., 2018), indicating a fitness cost. Thus, PGE_2 is crucial for *S. exigua* survivability, but the excessive amount may impose to reduced pupal mass and adult fecundity owing to a trade-off effect.

Acknowledgements
We thank Youngim Song for supplying materials and Al Baki Abdullah for technical guidance on the micro-injection of plasmids into young insect larvae.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Y.K.; Methodology: S.A., A.H., Y.K.; Formal analysis: S.A., A.H.; Investigation: S.A., A.H., Y.K.; Resources: Y.K.; Writing - original draft: S.A., A.H., Y.K.; Writing - review & editing: Y.K.; Visualization: S.A., A.H.; Supervision: Y.K.; Project administration: Y.K.; Funding acquisition: Y.K.

Funding
This work was supported by a grant (no. 2017R1A2133009815) from the National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning, Republic of Korea.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.207019.supplemental

References
Ahmed, S., Stanley, D. and Kim, Y. (2018). An insect prostaglandin E_2 synthase acts in immunity and reproduction. *Front. Physiol.* **9**, 1231. doi:10.3389/fphys.2018.01231

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye finding. *Anal. Biochem.* **72**, 248-254. doi:10.1016/0003-2697(76)90527-3

Burke, J. E. and Dennis, E. A. (2009). Phospholipase A_2 structure/function, mechanism, and signaling. *J. Lipid Res.* **50**, 237-242. doi:10.1194/jlr.R800033-JLR200

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L. et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611-622. doi:10.1373/clinchem.2008.112797

Dennis, E. A., Cao, J., Hsu, Y.-H., Magrioti, V. and Kokotos, G. (2011). Phospholipase A_2 enzymes: physical structure, biological function, disease implication, chemical inhibition and therapeutic intervention. *Chem. Rev.* **111**, 6130-6185. doi:10.1021/cr200085w

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987). Lipofection: a highly efficient lipid-mediated DNA transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 7413-7417. doi:10.1073/pnas.84.21.7413

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871-1875. doi:10.1126/science.294.5548.1871

Goh, H. G., Lee, S. G., Lee, B. P., Choi, K. M. and Kim, J. H. (1990). Simple mass-rearing of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), on an artificial diet. *Korean J. Appl. Entomol.* **29**, 180-183. doi:10.1007/springerreference_85461

Groen, C. M., Spracklen, A. J., Fagan, T. N. and Tootle, T. L. (2012). *Drosophila* Fascin is a novel downstream target of prostaglandin signaling during actin remodeling. *Mol. Biol. Cell.* **23**, 4567-4578. doi:10.1091/mbc.e12-05-0417

Hayaishi, O. (1991). Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D2 and E2. *FASEB J.* **5**, 2575-2581. doi:10.1096/fasebj.5.11.1907936

Hepat, R. and Kim, Y. (2012). *In vivo* transient expression for the functional analysis of polydnaviral genes. *J. Invertebr. Pathol.* **111**, 152-159. doi:10.1016/j.jip.2012.07.025

Hwang, J., Park, Y., Lee, D. and Kim, Y. (2013). An entomopathogenic bacterium, *Xenorhabdus nematophila*, suppresses expression of antimicrobial peptides controlled by Toll and IMD pathways by blocking eicosanoid biosynthesis. *Arch. Insect Biochem. Physiol.* **83**, 151-169. doi:10.1002/arch.21103

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402-408. doi:10.1006/meth.2001.1262

Lourenço, A. P., Martins, J. R., Bitondi, M. M. and Simões, Z. L. (2009). Trade-off between immune stimulation and expression of storage protein genes. *Arch. Insect Biochem. Physiol.* **71**, 70-87. doi:10.1002/arch.20301

Medeiros, M. N., Belmonte, R., Soares, B. C., Medeiros, L. N., Canetti, C., Freire-de-Lima, C. G., Maya-Monteiro, C. M., Bozza, P. T., Almeida, I. C., Masuda, H. et al. (2009). Arrest of oogenesis in the bug *Rhodnius prolixus* challenged with the fungus *Aspergillus niger* is mediated by immune response-derived PGE_2. *J. Insect Physiol.* **55**, 150-157. doi:10.1016/j.jinsphys.2008.10.019

Miller, J. S. (2005). Eicosanoids influence *in vitro* elongation of plasmatocytes from the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **59**, 42-51. doi:10.1002/arch.20052

Milton, A. S. and Wendlandt, S. (1971). Effects on body temperature of prostaglandins of the A, E and F series on injection into the third ventricle of unanaesthetized cats and rabbits. *J. Physiol.* **218**, 325-336. doi:10.1113/jphysiol.1971.sp009620

Park, Y., Kim, Y., Yi, Y. and Han, S. (1998). Optimal storage conditions of the entomopathogenic nematode, *Steinernema carpocapsae*. *Korean J. Appl. Entomol.* **3**, 10-16.

Park, Y., Aliza, A. R. N. and Stanley, D. (2005). A secretory PLA_2 associated with tobacco hornworm hemocyte membrane preparations acts in cellular immune reactions. *Arch. Insect Biochem. Physiol.* **60**, 105-115. doi:10.1002/arch.20086

Park, J., Stanley, D. and Kim, Y. (2014). Roles of peroxinectin in PGE_2-mediated cellular immunity in *Spodoptera exigua*. *PLoS ONE* **9**, e105717. doi:10.1371/journal.pone.0105717

Park, Y., Kang, S., Sadekuzzaman, M., Kim, H., Jung, J. K. and Kim, Y. (2017). Identification and bacterial characteristics of *Xenorhabdus hominickii* ANU101 from an entomopathogenic nematode, *Steinernema monticolum*. *J. Invertebr. Pathol.* **144**, 74-87. doi:10.1016/j.jip.2017.02.002

Park, Y., Kumar, S., Kanumuri, R., Stanley, D. and Kim, Y. (2015). A novel calcium-independent cellular PLA_2 acts in insect immunity and larval growth. *Insect Biochem. Mol. Biol.* **66**, 13-23. doi:10.1016/j.ibmb.2015.09.012

Sadekuzzaman, M., Gautam, N. and Kim, Y. (2017). A novel calcium-independent phospholipase A_2 and its physiological roles in development and immunity of a lepidopteran insect, *Spodoptera exigua*. *Dev. Comp. Immunol.* **77**, 210-220. doi:10.1016/j.dci.2017.08.014

SAS Institute, Inc. (1989). *SAS/STAT User's Guide*. Cary, NC: SAS Institute.

Scarpati, M., Qi, Y., Govind, S. and Singh, S. (2019). A combined computational strategy of sequence and structural analysis predicts the existence of a functional eicosanoid pathway in *Drosophila melanogaster*. *PLoS ONE* **14**, e0211897. doi:10.1371/journal.pone.0211897

Schwenke, R. A., Lazzaro, B. P. and Wolfner, M. F. (2016). Reproduction-immunity trade-offs in insects. *Annu. Rev. Entomol.* **61**, 239-256. doi:10.1146/annurev-ento-010715-023924

Shafeeq, T., Ahmed, S. and Kim, Y. (2018). Toll immune signal activates cellular immune response via eicosanoids. *Dev. Comp. Immunol.* **84**, 408-419. doi:10.1016/j.dci.2018.03.015

Shrestha, S. and Kim, Y. (2009). Various eicosanoids modulate the cellular and humoral immune responses of the beet armyworm, *Spodoptera exigua*. *Biosci. Biotechnol. Biochem.* **73**, 2077-2084. doi:10.1271/bbb.90272

Smith, W. L., Marnett, L. J. and DeWitt, D. L. (1991). Prostaglandin and thromboxane biosynthesis. *Pharmacol. Ther.* **49**, 153-179. doi:10.1016/0163-7258(91)90054-P

Spracklen, A. J., Kelpsch, D. J., Chen, X., Spracklen, C. N. and Tootle, T. L. (2014). Prostaglandins temporally regulate cytoplasmic actin bundle formation during *Drosophila* oogenesis. *Mol. Biol. Cell* **25**, 397-411. doi:10.1091/mbc.e13-07-0366

Stanley, D. (2006). Prostaglandins and other eicosanoids in insects: biological significance. *Annu. Rev. Entomol.* **51**, 25-44. doi:10.1146/annurev.ento.51.110104.151021

Stanley, D. and Kim, Y. (2014). Eicosanoid signaling in insects: from discovery to plant protection. *CRC. Crit. Rev. Plant Sci.* **33**, 20-63. doi:10.1080/07352689.2014.847631

Stanley, D. and Kim, Y. (2019). Prostaglandins and other eicosanoids in insects: biosynthesis and biological actions. *Front. Physiol.* **9**, 1927. doi:10.3389/fphys.2018.01927

Stanley-Samuelson, D. W., Peloquin, J. J. and Loher, W. (1986). Egg-laying in response to prostaglandin injections in the Australian field cricket, *Teleogryllus commodus*. *Physiol. Entomol.* **11**, 213-219. doi:10.1111/j.1365-3032.1986.tb00408.x

Tootle, T. L., Williams, D., Hubb, A., Frederick, R. and Spradling, A. (2011). *Drosophila* eggshell production: identification of new genes and coordination by Pxt. *PLoS ONE* **6**, e19943.

Umamaheswaran, S., Dasari, S. K., Yang, P., Lutgendorf, S. K. and Sood, A. K. (2018). Stress, inflammation, and eicosanoids: an emerging perspective. *Cancer Metastasis Rev.* **37**, 203-211. doi:10.1007/s10555-018-9741-1

Xiao, Q. L., Zhou, Y. J., Zhang, Z. F. and He, J. L. (2002). *In vitro* and *in vivo* transient expression in insect cells mediated by the cationic liposomes DDAB/DOPE. *Int. J. Indust. Entomol.* **4**, 57-62.

Yajima, M., Takada, M., Takahashi, N., Kikuchi, H., Natori, S., Oshima, Y. and Kurata, S. (2003). A newly established in vitro culture using transgenic *Drosophila* reveals functional coupling between the phospholipase A_2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. *Biochem. J.* **371**, 205-210. doi:10.1042/bj20021603

Zhang, L., Ringbauer, J. A., Jr., Goodman, C. L., Reall, T., Jiang, X. F. and Stanley, D. (2018). Prostaglandin-mediated recovery from bacteremia delays larval development in fall armyworm, *Spodoptera frugiperda*. *Arch. Insect Biochem. Physiol.* **97**, 21444. doi:10.1002/arch.21444