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Biosynthetic pathway of arachidonic acid in *Spodoptera exigua* in response to bacterial challenge

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ABSTRACT

Eicosanoids play crucial roles in mediating insect immune responses. In vertebrates, phospholipase A_2 (PLA_2) releases arachidonic acid (AA) from phospholipids (PLs) for biosynthesis of various eicosanoids. However, little AA is found in PLs of lepidopteran insects. *Spodoptera exigua*, a lepidopteran insect, is known to use eicosanoids to mediate immunity. Although AA was not detected in PLs of hemocytes and fat body (two immune tissues) of naïve larvae, it was detected at small but significant level after bacterial infection, suggesting induction of AA biosynthesis for immunity. Based on a mammalian AA biosynthetic pathway, this study hypothesizes that AA is synthesized from C18 polyunsaturated fatty acid (PUFA) precursor by subsequent desaturation and elongation reactions because PLs of *S. exigua* larvae are rich in linoleic acid. After inhibiting PLA_2 activity to prevent release of free fatty acids, different PUFA precursors were injected to *S. exigua* larvae followed by assessment of eicosanoid-mediated cellular immune response. ω-6 PUFAs were effective in inducing immune response whereas α-linolenic acid (an ω-3 PUFA) was not. Several fatty acyl desaturases (SeDESs) have been predicted from *S. exigua* transcriptomes. Specific inhibitors against Δ5 or Δ6 DESs inhibited eicosanoid-mediated immune responses. Furthermore, RNA interference (RNAi) specific to Δ5 or Δ6 DES genes significantly suppressed eicosanoid-mediated immune responses. Four very long chain fatty acid elongase genes (*SeEloV-A* ~ *SeEloV-D*) were predicted. Among respective RNAi treatments of these genes, only one RNAi treatment specific to type 5 elongase (*SeEloV-B*) suppressed eicosanoid-mediated immune response. These results suggest that *S. exigua* larvae can synthesize AA from linoleic acid via Δ5- and Δ6-desaturations by SeDESs along with chain elongation by SeEloV-B. Finally, this study showed significant fitness cost of uncontrolled AA biosynthesis. AA injection alone without bacterial challenge significantly induced both cellular and humoral immune responses. This unnecessary energy expense due to free AA resulted in reduced pupal size and decreased adult egg production. The detrimental effect of free AA explains physiological significance of little AA content in lepidopteran insects except for life-or-death situation such as pathogen infection.

1. Introduction

Eicosanoids are a group of oxygenated C20 polyunsaturated fatty acids (PUFAs). In mammals, arachidonic acid (AA, 20:4n-6), dihomo-γ-linolenic acid (20:3n-6), and eicosapentaenoic acid (20:5n-3) are used as C20 precursors for eicosanoid biosynthesis (Funk, 2001). Among these precursors, AA is a main precursor that is mostly esterified at *sn-2* position of cellular phospholipids (PLs) of cell membranes and internal membrane structures such as endoplasmic reticulum (Borkman et al., 1993). Phospholipase A_2 (PLA_2) hydrolyzes AA from PLs, leaving a free AA and a lysophospholipid in the membrane. AA is then oxygenated by cyclooxygenase (COX), lipoxygenase (LOX), or epoxidase (EPX) into prostaglandins (PGs), leukotrienes (LTs), or epoxyeicosatrienoic acids (EETs), respectively. These eicosanoids can act as autocrine or paracrine signals in mammalian physiological processes such as renal function and in pathophysiology including inflammation (Dennis and Norris, 2015). In insects, eicosanoids can also mediate various physiological processes including metabolism, immunity, reproduction, and excretion (Stanley and Kim, 2014).

PGs are fundamental mediators in insect cellular or humoral immunity. PGs can stimulate hemocyte behaviors such as micro-aggregation and nodule formation upon microbial challenge (Miller et al., 1994). PGs can also induce oenocytoid lysis to release inactive prophenoloxidase to plasma for its activation by serine proteases via a cell surface G-protein-coupled receptor (Shrestha and Kim, 2008; Shrestha et al., 2011). Especially, PGE_2 can induce specific immune activities

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including hemocyte spreading behavior (Srikanth et al., 2011), mobilization of sessile hemocytes (Park and Kim, 2000), and induction of antimicrobial peptide (AMP) gene expression (Yajima et al., 2003; Shrestha and Kim, 2007). Apart from immunity, PG has been shown to mediate insect reproduction in some insects (Loher, 1979). In the Australian field cricket, *Teleogryllus commodus*, PGE_2 is synthesized in female spermathecae by PG-synthetic enzymes transferred via spermatophores from males and stimulates egg-laying behavior of mated females (Stanley-Samuelson et al., 1986). PGs also mediate egg development in *Drosophila melanogaster* by coordinating expression of genes encoding egg shell proteins and stimulating material transfer from nurse cells to growing oocytes (Tootle and Spradling, 2008). In *Spodoptera exigua*, a lepidopteran insect, PGE_2 is required for choriogenesis of growing oocytes (Al Baki and Kim, 2019). PGs, not LTs or EETs, can modulate fluid secretion in mosquito Malpighian tubules (Petzel and Stanley-Samuelson, 1992) and forest ant, *Formica polyctena* (Van Kerkhove et al., 1995). Role of LTs in mediating insect immune responses has been elucidated from studies using pharmaceutical inhibitors specifically targeting catalytic activity of LOX (Park and Kim, 2000). Especially, a direct influence of LTB4 in *S. exigua* is known by mediating different cellular immune responses and stimulating AMP gene expression (Shrestha and Kim, 2009). Xu et al. (2015) have reported EET biosynthesis in *Culex quinquefasciatus* (the southern house mosquito) based on chemical detection of EET converted from AA in tissue lysate. Later, Xu et al. (2016) have fed mosquitoes with an orally active epoxide hydrolase inhibitor and found that midgut EET concentrations (especially 11,12-EET) are increased, leading to increased bacterial loads in female mosquitoes along with increased AMP gene expression. These findings indicate that all three major eicosanoid groups (PGs, LTs, and EETs) can act in insect physiological processes.

Although physiological processes mediated by eicosanoids are well known in insects, their biosynthetic pathways remain unclear. Scarpati et al. (2019) have proposed biosynthetic machineries for these three kinds of eicosanoids from *Drosophila* genome. Indeed, three different PLA_2s and two different COX-like peroxinectin genes associated with eicosanoid biosynthesis in *S. exigua* have been identified (Park et al., 2014, 2015; Sadekuzzaman et al., 2017; Vatanparast et al., 2018). However, *S. exigua* larvae that exhibit eicosanoid-mediating immune response possesses little AA and other C20 precursors (Kim et al., 2016). The lack of C20 PUFAs is commonly known in many terrestrial insects (Stanley-Samuelson et al., 1988).

Regarding biosynthesis of PUFAs, plants can synthesize linoleic acid (18:2n-3) and α-linolenic acid (18:3n-3). However, they cannot produce longer-chain PUFAs (Ruiz-Lopez et al., 2015). In contrast, most animals cannot synthesize linoleic acid (18:2n-3) or α-linolenic acid (18:3n-3), although they can metabolize these PUFAs into longer chain PUFAs (Wymann and Schneiter, 2008). However, Dwyer and Blomquist (1981) have shown that the injected ^{14}C-labeled acetate is incorporated into γ-linolenic acid (18:2n-6) in adult American cockroach, *Periplaneta americana*, suggesting possible biosynthetic activity of C18 PUFA in insects. Furthermore, Blomquist et al. (1991) have found that only some insects can biosynthesize 18:2n-6 and express a novel Δ12 desaturase responsible for inserting a double bond into 18:1n-9, yielding 18:2n-6. With similar approaches, Stanley-Samuelson (1994) have summarized that some species of Heteroptera, Isoptera, and Orthoptera can biosynthesize 18:2n-6 while most insect species are incapable of 18:2n-6 biosynthesis. Malcicka et al. (2018) explained the evolution of 18:2n-6 biosynthesis in some insects by dual function of some Δ9 desaturase containing Δ12 desaturase activity.

Insects exhibit biosynthetic abilities of long chain PUFAs from C18 precursors originated from diet or *de novo* synthesis. In the wax moth (*Galleria mellonella*) incapable of C18 PUFA biosynthesis, dietary requirement of PUFAs was investigated by supplementing fatty acid-free diet with increasing amounts of 18:3n-3 to produce normal adults (Dadd, 1983). Without PUFA supplementation, larvae were unable to develop to adults. However, PUFA addition rescued larvae to become adults, in which 18:3n-3 accounted for 6–27% of total phospholipid fatty acids and 20:5n-3 accounted for 0.4–3%, suggesting that this species can elongate and desaturate dietary 18:3n-3 to 20:5n-3 (Dadd, 1983). Another evidence that lepidopteran insects can metabolize C18 PUFAs to C20 PUFAs was demonstrated in white butterfly, *Pieris brassicae*, in which larvae could develop to adults under 18:3n-3 supplementation and subsequent adults possessed 20:3n-3 and 20:3n-3 (Parnanen and Turunen, 1987).

AA biosynthesis in insects was first reported in the American cockroach (*P. americana*) know to be capable of biosynthesizing C18 PUFAs (Jurenka et al., 1987), in which injected ^{14}C-acetate was metabolized into different C20 PUFAs including 20:4n-6. In *G. mellonella*, ^{14}C-labeled 18:2n-6 was metabolized into AA after injection, but not ^{14}C-labeled 18:2n-3 (Stanley-Samuelson et al., 1987). However, genetic factors associated with AA biosynthesis in insects remained unknown.

This study hypothesizes that AA is biosynthesized from C18 precursor by chain elongation and desaturation in *S. exigua*. Thus, the objective of this study was to test this hypothesis and determine genetic factors associated with reactions to synthesize AA. To this end, candidate genes were predicted from *S. exigua* transcriptomes and functional assays were performed by RNA interference (RNAi) to confirm changes of eicosanoid-mediated immune responses in *S. exigua*.

2. Materials and methods

2.1. Insect rearing and bacterial culture

Larvae of *S. exigua* were reared on an artificial diet as described previously (Shrestha et al., 2011) at 25 °C with relative humidity of 60 ± 5% and 16 h/8 h of light/dark photoperiod. Adults were fed 10% sucrose solution. Larvae of different instars (L1-L5) were discriminated based on their head width (Goh et al., 1990). *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) cells were cultured overnight in LB medium (Difco, Sparks, MD, USA) at 37 °C in a shaking (200 rpm) incubator and used in nodulation assay.

2.2. Chemicals

Linoleic acid [LA: (9*Z*,12*Z*)-octadeca-9,12-dienoic acid], α-linolenic acid [ALA: (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid], γ-linolenic acid [GLA: (6*Z*,9*Z*,12*Z*)-octadeca-6,9,12-trienoic acid], eicosadienoic acid [EDA: (11*Z*,14*Z*)-eicosa-11,14-dienoic acid], dihomo-γ-linolenic acid [DGLA: (8*Z*,11*Z*,14*Z*)-eicosa-8,11,14-trienoic acid], arachidonic acid [(5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,11,14-tetraenoic acid], dexamethasone [DEX: (11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], sesamin [5,5'-(1*S*,3a*R*,4*S*,6a*R*)-tetrahydro-1*H*,3*H*-furo [3,4-c] furan-1,4-diylbis (1,3-benzodioxole)], SC-26196 [α,α-diphenyl-4-[(3-pyridinylmethylene) amino]-1-piperazinepentanenitrile], and PluriSln 1 [4-pyridinecarboxylic acid 2-phenylhydrazide] were purchased from Sigma-Aldrich Korea (Seoul, Korea). They were dissolved in dimethyl sulfoxide (DMSO) to obtain different concentrations. Anticoagulant buffer (ACB, pH 4.5) was prepared with 186 mM NaCl, 17 mM Na_2EDTA, and 41 mM citric acid.

2.3. Hemocyte-spreading assay using in situ fluorescence

Hemolymph (~200 μL) was collected in 800 μL of cold ACB from five L5 larvae by cutting prolegs and incubated on ice for 30 min. After incubation, hemolymph was centrifuged at 800 × *g* for 5 min at 4 °C to separate hemocytes as pellet. Supernatant plasma was discarded and pellet was re-suspended in 500 μL of filter-sterilized TC-100 insect cell culture medium (Welgene, Daegu, Korea). On a glass coverslip placed in a moist chamber, 10 μL of hemocyte suspension was applied and placed in a dark condition. Hemocytes were then fixed with 4% paraformaldehyde (filter-sterilized) for 10 min at 25 °C and then washed thrice with filter-sterilized 100 mM phosphate-buffered saline (PBS, pH

Table 1
Fatty acid compositions of neutral lipid (NL), glycolipid (GL) and phospholipid (PL) in fifth instar larvae of *S. exigua*.

Fatty acids	Content (%)			
	NL	GL	PL	*P* value (*F* test)
Hemocytes				
Myristic acid (14:0)	0.00	2.45 ± 0.08	0.00	0.0000
Palmitic acid (16:0)	24.52 ± 1.02	31.22 ± 5.7	23.67 ± 6.82	0.1578
Palmitoleic acid (16:1, Δ^9)	1.41 ± 0.02	0.00	0.08 ± 0.00	0.0023
Stearic acid (18:0)	2.26 ± 0.63	8.92 ± 3.23	0.00	0.0035
Oleic acid (18:1, Δ^9)	22.66 ± 0.32	15.13 ± 6.98	11.86 ± 0.82	0.0092
Linoleic acid (18:2, $\Delta^{9,12}$)	26.82 ± 13.88	25.62 ± 6.5	35.86 ± 15.62	0.0461
γ-Linolenic acid (18:3, $\Delta^{6,9,12}$)	0.00	0.00	1.23 ± 0.05	0.0000
Eicosanoic acid (20:0)	1.40 ± 0.01	2.3 ± 1.5	2.44 ± 1.5	0.1275
Fat body				
Myristic acid (14:0)	6.74 ± 1.64	0.69 ± 0.16	0.32 ± 0.21	0.0045
Myristoleic acid (14:1, Δ^9)	0.13 ± 0.03	0.00	0.00	0.0000
Palmitic acid (16:0)	32.52 ± 1.64	25.12 ± 1.95	20.83 ± 1.76	0.0821
Palmitoleic acid (16:1, Δ^9)	4.21 ± 1.53	0.83 ± 0.16	1.16 ± 0.67	0.0038
Stearic acid (18:0)	51.47 ± 1.93	11.88 ± 0.55	27.76 ± 12.43	0.0004
Oleic acid (18:1, Δ^9)	32.15 ± 5.34	10.52 ± 2.22	14.22 ± 1.45	0.0048
Linoleic acid (18:2, $\Delta^{9,12}$)	25.12 ± 2.55	35.41 ± 1.74	17.80 ± 2.70	0.0023
γ-Linolenic acid (18:3, $\Delta^{6,9,12}$)	0.00	2.25 ± 0.88	0.00	0.0000
Eicosanoic acid (20:0)	1.51 ± 0.42	3.83 ± 1.36	2.16 ± 0.89	0.0512
11-Eicosenoic acid (20:1, Δ^{11})	4.94 ± 1.46	0.00	1.46 ± 0.61	0.0031

Table 2
Changes in total fatty acid compositions of fat body phospholipid (PL) in fifth instar larvae of *S. exigua*.

Fatty acids	Fatty acid content (%) in PL		*P* value (*t*-test)
	Naïve	Bacterial challenge	
Hemocytes			
Palmitic acid (16:0)	22.51 ± 5.78	20.31 ± 3.84	0.091
Stearic acid (18:0)	0.52 ± 0.00	0.48 ± 1.22	0.121
Oleic acid (18:1, Δ^9)	12.33 ± 0.91	13.53 ± 1.52	0.077
Linoleic acid (18:2, $\Delta^{9,12}$)	32.51 ± 9.21	37.42 ± 6.37	0.116
γ-linolenic acid (18:3, $\Delta^{6,9,12}$)	1.02 ± 0.04	1.98 ± 0.52	0.021
Eicosanoic acid (20:0)	2.83 ± 0.37	2.77 ± 0.37	0.173
Eicosadienoic acid (20:2, $\Delta^{11,14}$)	0.00	0.52 ± 0.00	0.000
Fat body			
Stearic acid (18:0)	48.17 ± 4.72	49.07 ± 3.29	0.078
Oleic acid (18:1, Δ^9)	21.16 ± 1.75	23.09 ± 3.26	0.116
Linoleic acid (18:2, $\Delta^{9,12}$)	17.80 ± 2.70	15.21 ± 2.39	0.093
α-linolenic acid (18:3, $\Delta^{9,12,15}$)	1.27 ± 0.52	1.22 ± 0.37	0.172
Eicosadienoic acid (20:2, $\Delta^{11,14}$)	0.00	1.25 ± 0.35	0.024
Arachidonic acid (20:4, $\Delta^{5,8,11,14}$)	0.00	0.20 ± 0.07	0.029

7.4). Cells were then permeabilized with 0.2% Triton-X dissolved in PBS for 2 min at 25 °C and washed with PBS. After that, hemocytes were blocked using 10% bovine serum albumin (BSA) dissolved in PBS for 10 min at 25 °C and washed again with PBS. Cells were then incubated with fluorescein isothiocyanate (FITC)-tagged phalloidin (Thermo Fisher Scientific Korea, Seoul, Korea) in PBS for 60 min and washed thrice with PBS. Hemocyte nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) (Thermo Fisher Scientific Korea) dissolved in PBS and washed thrice with PBS. Hemocytes slides were then observed under a fluorescence microscope (DM2500, Leica, Wetzlar, Germany) at 400× magnification. Hemocyte-spreading was determined based on extension of F-actin outward the cell boundary of hemocyte.

2.4. Hemocyte nodulation assay

Three days old L5 (L5D3) larvae were surface-sterilized with 70% ethanol and nodulation assay was performed by injecting bacterial suspension (5×10^4 cells/larva). At 8 h after bacterial challenge, insect larvae were dissected to count nodules under a stereomicroscope (Stemi SV 11, ZEISS, Jena, Germany) at 50× magnification. LA, ALA, GLA, EDA, EDA, AA, or DEX was injected to L5D3 larvae at a dose of 100 μg per larva along with bacterial injection. Specific desaturase inhibitors (sesamin, SC-26196, or PluriSln #1) were injected at a dose of 10 μg per larva along with bacteria suspension. Each treatment was performed in triplicate using ten insects per replicate.

2.5. Bioinformatics

Open reading frames (ORFs) of seven *S. exigua* desaturases (DESs) were retrieved from GenBank (www.ncbi.nlm.nih.gov) (accession numbers: KU755469, KU755474, KU755473, KU755471, KU755468, KU755475, and KU755472). These desaturases were termed SeDES-A ~ SeDES-G, respectively.

Very long chain fatty acid elongase (EvoL) genes were obtained from GenBank after screening transcriptome shotgun assembly (TSA taxid: 7107) of *S. exigua* using *Papilio xuthus* elongase (NM_001312325). Four BLAST contigs (GAOQ01017579.1, GARL01044491.1, GAOQ01017503.1, and GARL01041609.1) showing high identity (E value < 10^{-20}) were analyzed for ORFs and deposited at NCBI GenBank (accession numbers: ML025798, ML025799, ML025800, and ML025801). Predicted amino acid sequences of SeEloVs were aligned with other elongases (HsEloV1: NP_07372.1, PcEloV2: XP_025110341.1, FcEloV3: XP_021962857, HmEloV4: XP_021182691.1, VjEloV5: XP_022700704.1, HmEloV6: XP_021187985.1, and BmEloV7: XP_004924759.1) using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), a multiple sequence alignment program provided by European Bioinformatics Institution. Phylogenetic tree was generated by using Neighbor-joining method with Mega6 and Clustal W programs. Bootstrapping values were obtained with 1,000 repetitions to support branch and clustering. Protein domain was predicted using InterPro tool (https://www.ebi.ac.uk/interpro/) and Prosite (http://prosite.expasy.org/).

2.6. RNA extraction and cDNA preparation

Total RNAs were extracted from all developmental stages (egg, L1-L5, pupa, and adult) and tissues (hemocytes, fat body, and midgut) of *S. exigua* using Trizol reagent (Life Technologies, Carlsbad, CA, USA)

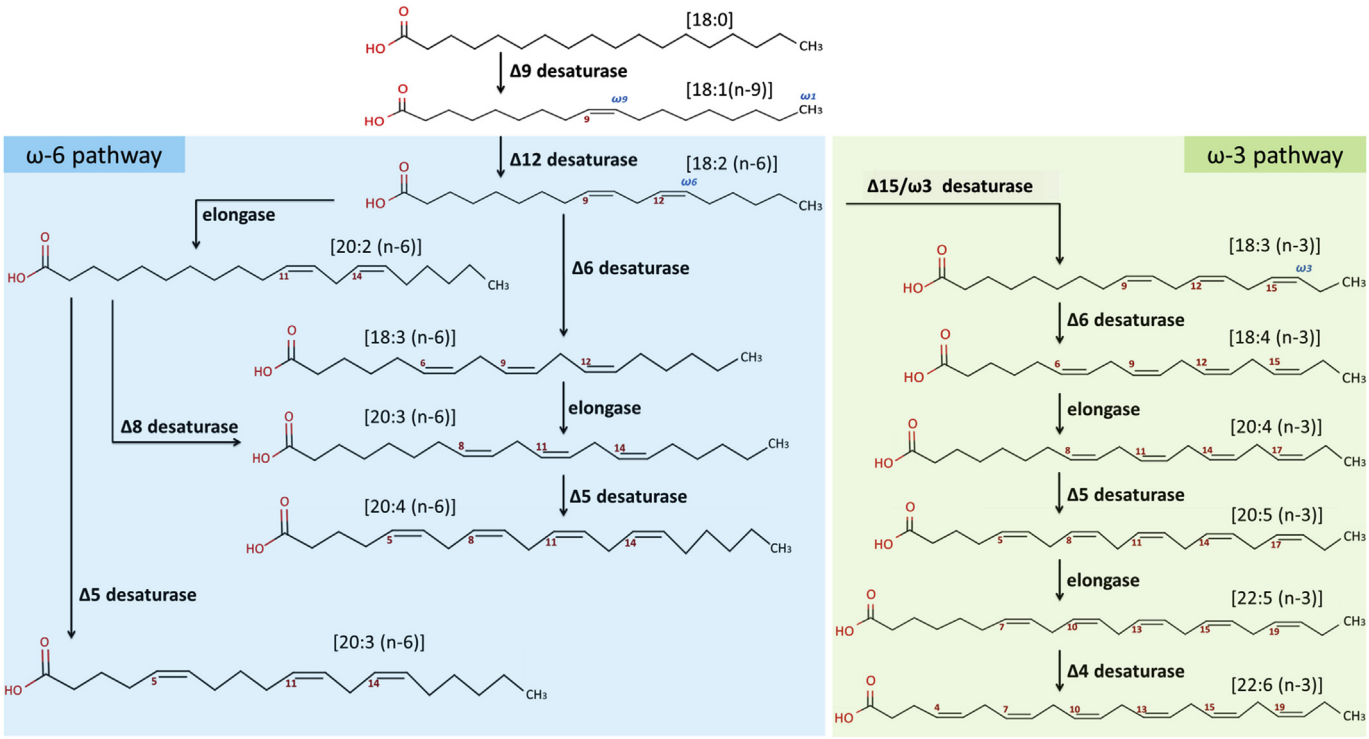


Fig. 1. A hypothesis of biosynthetic pathways of arachidonic acid (20:4(n-6)) and other long chain-polyunsaturated fatty acids (LC-PUFAs) from C18 PUFAs in insects.

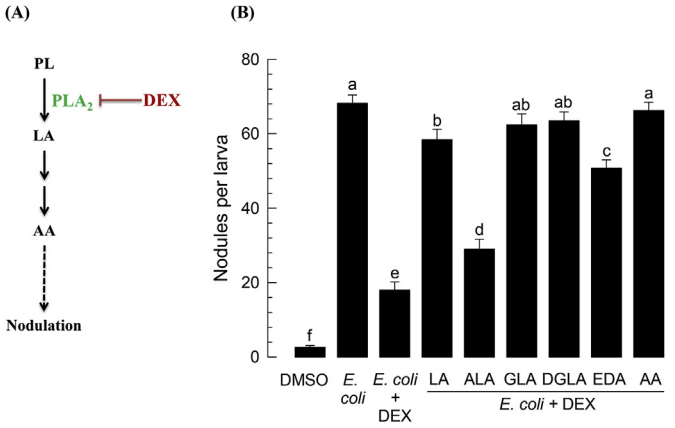


Fig. 2. Screening precursors for arachidonic acid (AA) biosynthesis in *S. exigua*. (A) A hypothetical AA biosynthetic pathway and its associated immune response. PLA_2 releases linoleic acid (LA) from phospholipid (PL). After a series of reactions, LA is changed into AA which is used for biosynthesis of various eicosanoids that mediate cellular immune response such as nodulation. (B) Precursor screening by measuring a rescue effect of additional candidate precursors on immunosuppression induced by PLA_2 inhibitor (dexamethasone: DEX). Immune response was measured by hemocyte nodule formation. Test precursors included LA, α-linolenic acid (ALA), γ-linolenic acid (GLA), dihomo-GLA (DGLA), cis-11,14-eicosadienoic acid (EDA), and AA. For immune challenge, *E. coli* (1.8×10^5 cells/larva) was injected to L5 larvae along with precursor compound (100 μg/larva). After 8 h of incubation at 25 °C, the number of formed nodules was counted. Each treatment was replicated with 10 larvae. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test).

following the manufacturer's instructions. For RNA extraction, different individual numbers were used for each developmental stage: ~500 individuals for eggs, ~50 larvae for L1-L2, ~20 larvae for L3-L4, and two individuals for L5, pupa, or adult. L5 larva was used for collecting different tissues. After extraction, total RNA was resuspended in nuclease-free water and quantified using a spectrophotometer (GeneQuant Pro, Amersham, Biosciences, Cambridge, England). cDNA synthesis from 1 μg of total RNA was performed using Maxime RT PreMix (Intron Biotechnology, Seoul, Korea) that contained oligo dT primer according to the manufacturer's instruction.

2.7. RT-qPCR of DES and EloV genes

DES and EloV genes were amplified using gene-specific primers (Table S1). PCR was performed with 35 cycles of denaturation at 94 °C for 1 min, annealing at 47 °C for 1 min, and extension at 72 °C for 1 min. PCR products were separated on 1% agarose gel by electrophoresis. RT-qPCR was performed on a qPCR machine (CFX Connect Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA) with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) and gene-specific primers used in RT-PCR. RT-qPCR was performed with 40 cycles of 15 s at 95 °C, 30 s at 50 °C, and 45 s at 72 °C. After PCR reactions, melting curves from 60 to 95 °C were obtained to confirm unique PCR products. *RL32*, a ribosomal protein, was used as a reference gene (Table S1). Each treatment was independently triplicated. The relative quantitative analysis method ($\Delta\Delta C_T$) was used to estimate relative mRNA expression levels of desaturase genes.

2.8. RNA interference of desaturase genes

Double stranded RNAs (dsRNAs) of DES and EloV genes were prepared using Megascript RNAi kit (Ambion, Austin, TX, USA) according to manufacturer's instruction. Briefly, DES gene fragments were produced by PCR using gene-specific primers containing T7 RNA polymerase promoter sequence at the 5' end. Sense and antisense-RNA strands were synthesized using T7 RNA polymerase at 37 °C for 4 h according to the method described by Park et al. (2015). The synthesized dsRNA was mixed with Metafectene PRO (Biontex, Plannegg, Germany) at 1:1 vol ratio and incubated at 25 °C for 30 min to form liposomes. About 900 ng of the dsRNA was then injected to each larval

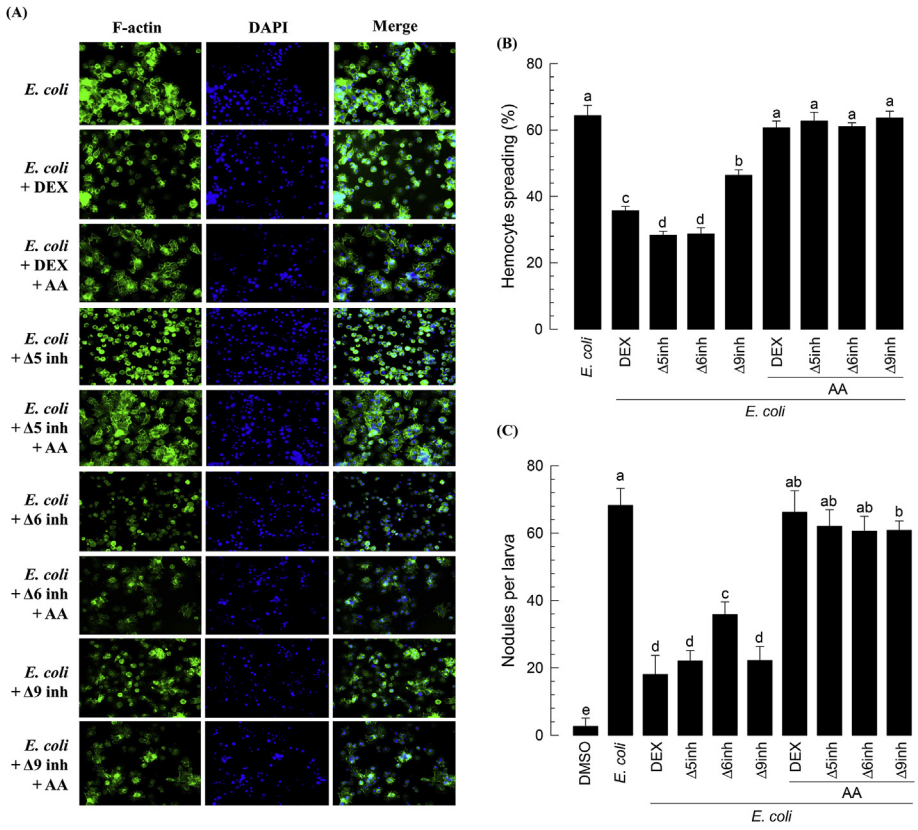


Fig. 3. Role of desaturases in arachidonic acid (AA) biosynthesis of *S. exigua*. Functional association of desaturases (DESs) with AA biosynthesis was assessed based on inhibitory activity of specific DES inhibitors against hemocyte-spreading behavior and nodule formation. Three specific inhibitors were used in this assay: sesamin (Δ5inh) for Δ5-DES, SC-26196 (Δ6inh) for Δ6-DES, and PluriSln 1 (Δ9inh) for Δ9-DES. Dexamethasone (DEX) was used for PLA_2 inhibitor to shutdown AA biosynthesis. All inhibitors were injected into L5 larvae at a dose of 10 μg/larva. To rescue the inhibitor effect, 10 μg of AA was co-injected with inhibitors in separate experiments. (A) Influence of DES inhibitors on hemocyte-spreading behavior. A6 h post incubation at 25 °C, hemocytes from treated larvae were collected for fluorescence observation of F-actin using FITC-labeled phalloidin. Nuclei were stained with DAPI. (B) Quantitative analysis of inhibitory effects of DES inhibitors on hemocyte-spreading behavior. Spread cells were confirmed from fluorescence assay by cytoplasmic extension along with F-actin growth. Each treatment was independently replicated three times. (C) Influence of DES inhibitors on nodule formation. Inhibitors were injected (10 μg/larva) into L5 larvae along with *E. coli* (1.8 × 105 cells/larva). In separate experiment, AA (10 μg/larva) was co-injected with inhibitors and bacteria to check the rescue effect on nodulation. Treated insects were incubated at 25 °C for 8 h and then assessed for nodule formation. Each treatment was triplicated with 10 larvae per replication. Different letters above standard error bars indicate significant difference among means at type I error = 0.05 (LSD test).

hemocoel. As a dsRNA control (dsCON), dsRNA specific to CpBV-ORF302, a viral gene (Park and Kim, 2011), was injected. Microinjection was performed using a microsyringe (Hamilton, Reno, NV, USA) equipped with a 26 gauge needle. At 0, 12 24, and 48 h post-injection (PI), RNAi efficacies in reducing target gene expression were measured by RT-qPCR as described above. At 24 h PI, treated larvae were used for immune challenge to determine nodulation. Each treatment was triplicated.

2.9. GC-MS analysis of fatty acids

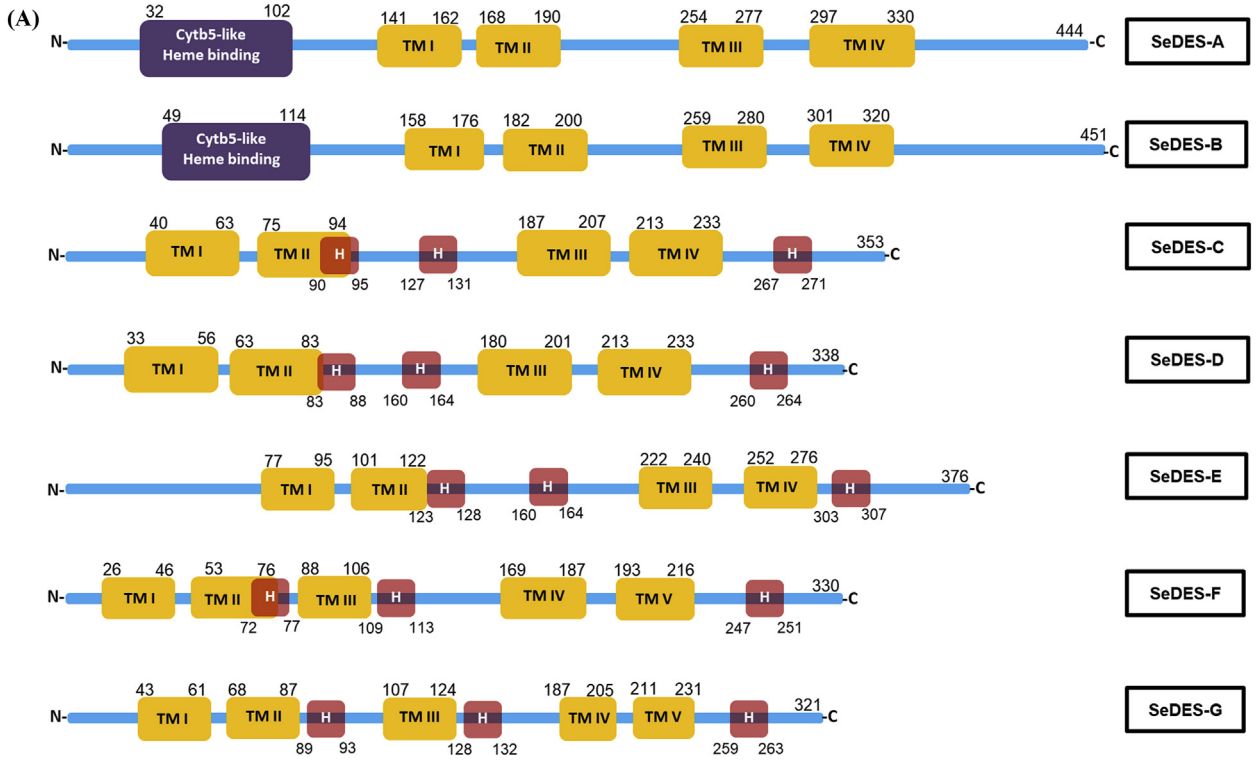
Total lipid was extracted from the fat body of L5 larva of *S. exigua* following published method (Folch, 1957). Briefly, fat body (~1 g) was collected from naïve or bacteria-challenged L5 larvae. For bacterial challenge, 5×10^4 cells of *E. coli* were injected to the larvae and incubated for 8 h at 25 °C. The fat body was then mixed with chloroform: methanol (2:1, v/v) with a homogenizer (Branson Ultrasonics, Danbury, CT, USA). The homogenate was then filtered using filter paper (Grade 1; Whatman, Little Chalfront, UK) and subject to fractionation of neutral lipid (NL), glycolipid (GL), and phospholipid (PL) using silica (70–230 mesh; Merck, Darmstadt, Germany) column (5.5 × 40 mm) chromatography. Different organic solvents were used for fractionation: chloroform for NL, acetone: methanol (9:1, v/v) for GL, and methanol for PL. Saponification and methylation followed MIDI method (http://midi-inc.com/pdf/Sherlock_MIS_Operating_Manual.pdf). Briefly, lipids were hydrolyzed with a basic saponification solution (45 g of sodium hydroxide, 150 mL of methanol, and 150 mL of H_2O) followed by heating at 100 °C for 25 min. A methylation solution (325 mL of 6 N HCl and 275 mL of methanol) was added to the specimen and heated at 80 °C for 10 min. After cooling down to room temperature, an extraction solution containing hexane: methyl tert-butyl ether (1:1, v/v) was added. The upper phase containing methylated free fatty acids was then analyzed in a GC-MS equipped with a DB-5MS column (30 m × 0.25 mm, 0.25 μm film thickness) (Agilent Technologies, Inc., Santa Clara, CA, USA). The oven temperature was maintained at 100 °C for 4 min and then increased at 3 °C per min to 240 °C. The oven was then maintained at this temperature (240 °C) for 15 min. The mobile phase gas was helium at flow rate of 0.7 mL/min. Injector temperature was set at 225 °C and ion source temperature was kept at 200 °C. Each sample was analyzed by injecting 1 μL sample with a split ratio of 20:1. Resulting peaks were identified based on retention times of known fatty acid methyl esters. Amounts of identified fatty acids were estimated based on peak area ratios.

2.10. Developmental assay after AA injection

AA was injected to L5D1 larvae of *S. exigua* at different doses (0–100 μg/larva) and incubated at 25 °C for 48 h. After the incubation period, the survival rate of insects was calculated. The experiment was triplicated with 10 insects in each replicate. In a separate experiment, 1 μg of AA was injected into L5D1 larvae of *S. exigua* and reared at 25 °C until pupation. Subsequently, pupal weight on the first day after pupation was obtained and oocyte number in each female ovary was counted. The experiment was triplicated with 10 insects in each replicate.

2.11. Statistical analysis

All assays were analyzed in one-way ANOVA by PROC GLM for continuous variables using SAS program (SAS Institute, 1989). Frequency data were analyzed by two-way Chi-square test using PROC FREQ of SAS program. Mortality data were subjected to arcsin transformation and used for ANOVA. The means were compared by least squared difference (LSD) test at Type I error = 0.05.



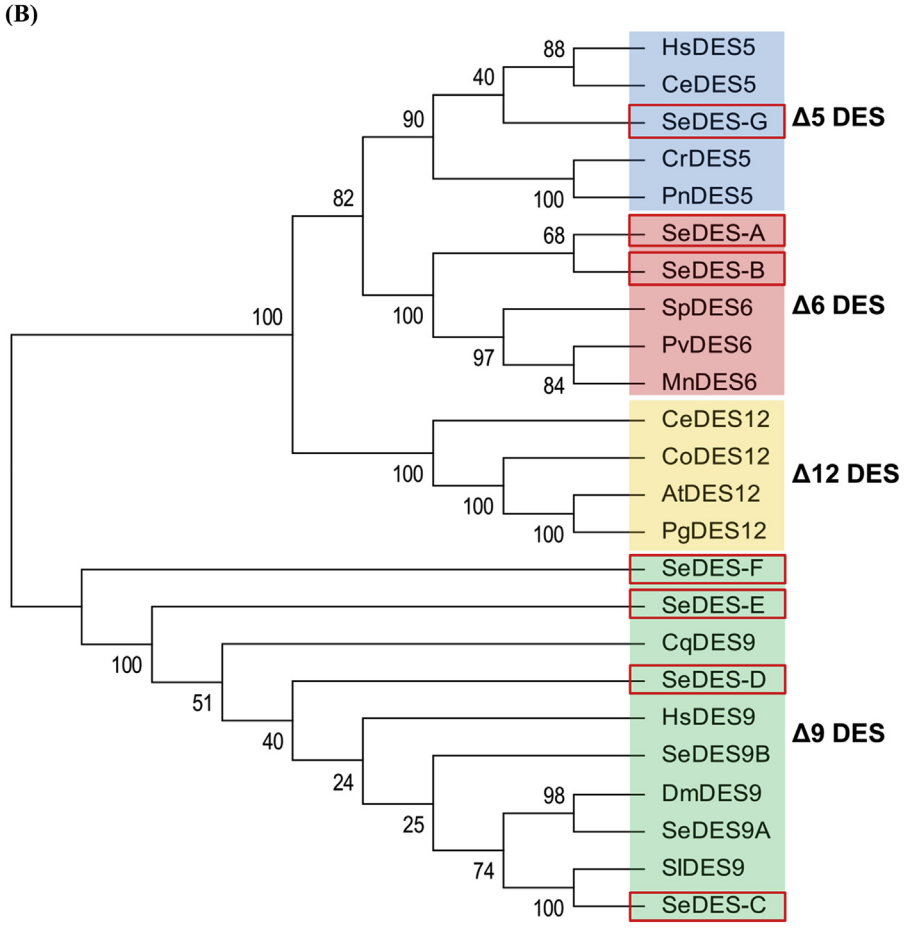


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Fig. 4. Prediction of fatty acid desaturases (SeDESs) of *S. exigua*. (A) Domain analysis of putative seven SeDESs. SeDES-A and Se-DES-B contain four transmembrane regions (TM I ~ TM IV) and a cytb5-like heme binding domain. SeDES-C ~ SeDES-E contain four TM domains and three conserved histidine boxes (H) characterized by HXXXXH, HXXHH, and HXXHH motifs. The other two desaturases (SeDES-F and SeDES-G) also contain three H domains. But they have five TM domains. Conserved domains were determined using InterPro tool (https://www.ebi.ac.uk/interpro/) and Prosite (http://prosite.expasy.org/) whereas other residues and motifs were predicted using several tools from DTU bioinformatics (www.cbs.dtu.dk/services/). (B) Phylogenetic analysis of *SeDES*s along with Δ5, Δ6, Δ9, and Δ12 DESs of other organisms. Amino acid sequences were retrieved from GenBank: HsDES5 (NP_037534), CeDES5 (NP_001255423.1), CrDES5 (ACO10922.1), PnDES5 (APH81338.1), PvDES6 (ALS03812.1), HsDES6 (NP_004256.1), MnDES6 (AMQ48726.1), SpDES6 (ANA07380.1), CeDES12 (NP_502560.1), CoDES12 (Q9SCG2.1), AtDES12 (NP_187819.1), PgDES12 (Q84VT2.2), CqDES9 (XP_001864524.1), HsDES9 (NP_005054.3), SeDES9A (AFO38464.1), SeDES9B (AFO38465.1), DmDES9 (NP_652731.1), and SlDES9 (AGH12218.1). The tree was constructed with Neighbor-joining method using MEGA6.0. Bootstrapping values on branches were obtained with 1,000 repetitions.

3. Results

3.1. De novo synthesis of AA upon bacterial challenge

Fatty acid compositions in different lipids of *S. exigua* larvae were analyzed to determine the amount of AA. Results are shown in Table 1. In both hemocytes and fat body, 10 different fatty acids were identified from GC-MS analysis, including four saturated and six unsaturated fatty acids. AA was not identified. More than 50% fatty acids were palmitic acid, stearic acid, oleic acid, and linoleic acid (LA) in three different lipid fractions (NL, GL, and PL) of both tissues. However, fatty acid compositions were different between hemocytes and fat body ($X^2 = 138.17$; df = 4; $P < 0.0001$). They were also different among three different lipid types in hemocytes ($F = 5.12$; df = 2, 47; $P < 0.0001$) and fat body ($F = 5.22$; df = 2, 47; $P < 0.0001$).

In PL, the most frequent fatty acid was LA in hemocytes of naïve larvae. LA was also rich in PL extracted from fat body. Upon bacterial challenge, AA accounted for 0.2% of total fatty acids in the fat body (Table 2). In addition, γ-linolenic acid (GLA) was significantly ($P < 0.05$) increased in hemocytes upon bacterial challenge. Eicosadienoic acid (EDA) was newly detected at 0.52–1.2% in both bacterial challenged tissues (fat body and hemocytes). In contrast, four main fatty acids (stearic acid, oleic acid, LA, α-linolenic acid) were not significantly ($P > 0.05$) changed in response to bacterial challenge.

3.2. LA is a precursor for AA biosynthesis

In vertebrates, AA is synthesized by desaturation and elongation from LA in ω-6 biosynthetic pathway (Fig. 1). LA is desaturated by Δ6 desaturase into GLA. Subsequent elongation and Δ5 desaturation lead to production of AA. Alternatively, Δ8 DES catalysis might participate in AA synthesis. Here, we hypothesize that PLA_2 releases precursor(s) of AA biosynthesis upon bacterial challenge. The precursor(s) will then undergo elongation/desaturation to become AA. To determine the precursor(s) in the AA biosynthetic pathway of *S. exigua* after bacterial challenge, different fatty acids were added to larvae treated with PLA_2 inhibitor (Fig. 2A). Larvae treated with PLA_2 inhibitor did not show nodule formation in response to bacterial challenge (Fig. 2B). However, AA addition rescued such immunosuppression. This rescue effect was also observed after treatment with LA, GLA, dihomo-GLA, or EDA, although the effect was not as much as that with α-linolenic acid (ALA). This suggests that AA biosynthesis follows ω-6 fatty acid biosynthesis from LA.

3.3. Desaturases are associated with immune responses mediated by AA

AA biosynthesis from LA requires catalytic activities of different desaturases. Inhibitors specific to different desaturases were used for treatment to assess hemocyte behavior mediated by AA (Fig. 3). Without any treatment, hemocytes could spread on slide glass by extending cytoplasm along with growth of F-actin (Fig. 3A). However, treatment with PLA_2 inhibitor ('DEX') significantly suppressed such hemocyte-spreading behavior (Fig. 3B). Similar inhibitory activities were observed after treatment with inhibitor specific to Δ5, Δ6, or Δ9 desaturase, although Δ5 and Δ6 desaturase inhibitors had more potent inhibitory activities than Δ9 desaturase inhibitor. Inhibitory activities of these specific inhibitors were also observed in results of cellular immune response measured by nodule formation (Fig. 3C). All inhibitory activities of desaturase inhibitors were significantly ($P < 0.05$) rescued by addition of AA.

3.4. Desaturases of S. exigua and their association with immune responses mediated by AA

In *S. exigua*, two desaturases (*SeDES-9A* and *SeDES-9B*) had been annotated (AFO38464.1 and AFO38465.1). This study newly added seven other desaturases (*SeDES-A* ~ *SeDES-G*) (Fig. 4). Protein domain analysis of these desaturases predicted four to five transmembrane helices (TM-TM5), three conserved histidine box motifs (H1–H3), and cytochrome *b*5-like heme-binding domain in *SeDES-A* and *SeDES-B* (Fig. 4A). Phylogenetic analysis showed the following clusterings: *SeDES-A* and *SeDES-B* clustered with Δ6 desaturases; *SeDES-C* ~ *SeDES-F* clustered with Δ9 desaturases; and *SeDES-G* clustered with Δ5 desaturases (Fig. 4B).

Most SeDES genes were expressed in different developmental stages, although their expression levels were significantly ($P < 0.05$) different (Fig. 5A). All *SeDES*s were expressed in eggs except for *SeDES-A* and *SeDES-C*. Most of them were highly expressed in adults except *SeDES-D*. Three desaturases (*SeDES-B*, *SeDES-F*, and *SeDES-G*) were highly expressed in most developmental stages compared to others based on their relative expression levels.

Bacterial challenge up-regulated expression levels of these desaturase genes in three different larval tissues (Fig. 5B). Immune challenge increased expression levels of most desaturases (except *SeDES-E*) in hemocytes. Most desaturases (except *SeDES-F*) exhibited up-regulation in fat body and midgut. Especially, *SeDES*s clustered with Δ5 and Δ6 desaturases exhibited the inducible expression patterns in response to bacterial challenge.

RNAi using dsRNA injection was effective in suppressing expression levels of SeDES genes (Fig. 6A). Injection of control dsRNA ('dsCON') did not show any significant change in expression levels of *SeDES*s (see box in Fig. 6A). However, gene-specific dsRNAs significantly ($P < 0.05$) suppressed target mRNA levels at 12–48 h after injection. Under RNAi conditions, nodule formation mediated by AA was assessed. All dsRNA treatments suppressed nodule formation in response to bacterial challenge (Fig. 6B). AA addition significantly ($P < 0.05$) rescued the suppressed immune response in all treatments. These results suggest that desaturases including Δ5-DES and Δ6-DES are associated with AA-mediating cellular immune response.

3.5. Elongases of S. exigua and their association with immune responses mediated by AA

For chain elongation of LA to produce C20 PUFAs, very long fatty acid chain elongase (EloV) might be necessary to work together with DESs. Four EloV genes (*SeEloV-A* ~ *SeEloV-D*) were obtained from *S. exigua* transcriptome as shown in Fig. 7. Multiple sequence alignment of predicted amino acid sequences of these four SeEloVs with other species elongases indicated that all four *SeEloV*s contained several similar motifs (KXXEXXDT, HXXMYXYY, TXXQXXQ) and a histidine box

(A)

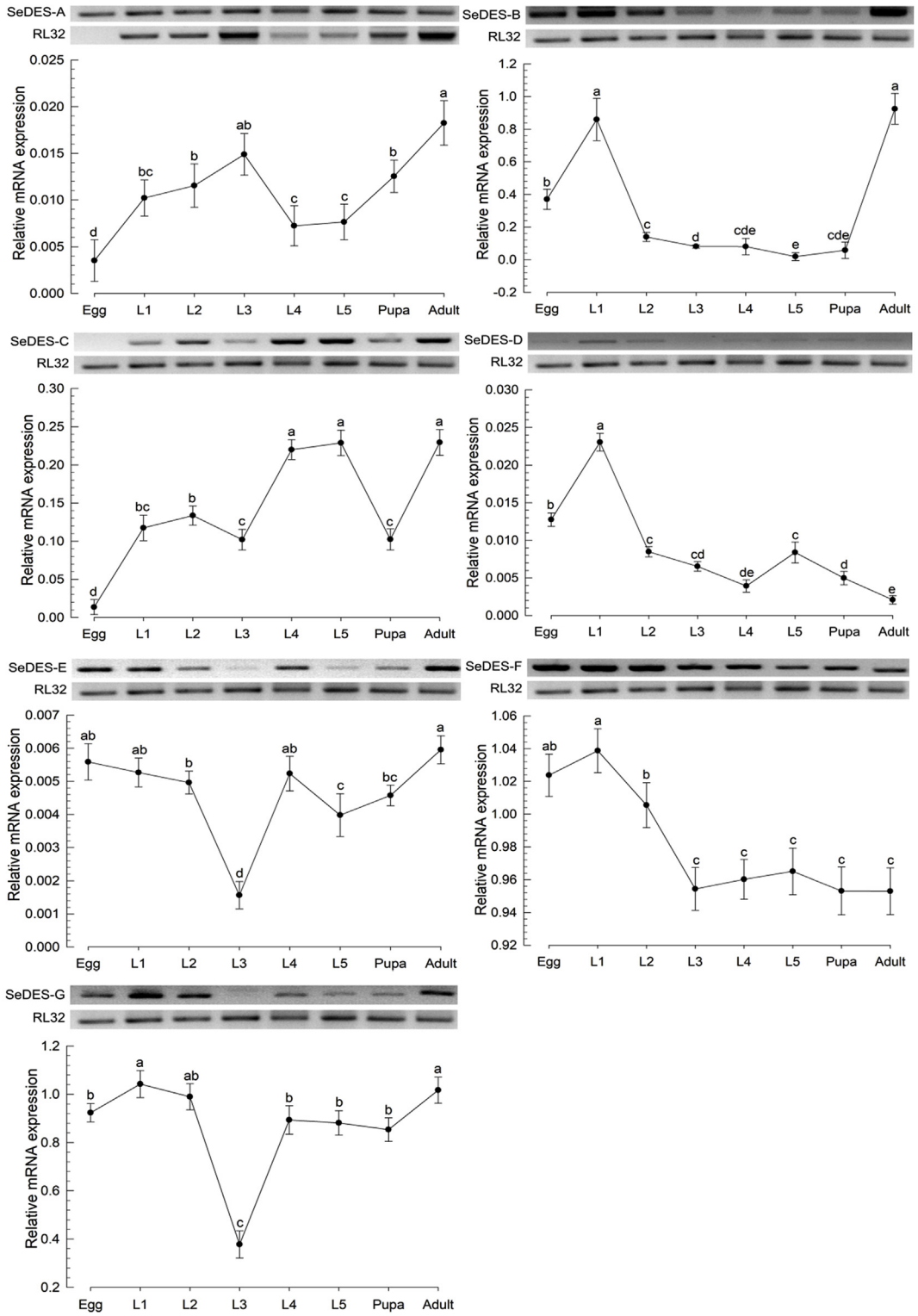


Fig. 5. Expression profiles of seven desaturases (*SeDES-A* ~ *SeDES-G*) of *S. exigua*. (A) Their expression patterns in different developmental stages [egg, larval instars ('L1-L5'), pupa, and adult] of *S. exigua.* Each measurement was replicated three times with independent biological samples. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test). (B) Influence of immune challenge on *SeDES* expression in three different larval tissues [hemocyte ('HC'), fat body ('FB'), and midgut ('GUT')]. Expression levels were compared between naïve and immune-challenged insects. Immune challenge was performed by injecting each L5 larva with 1.8×10^5 cells of *E. coli*. After incubation at 25 °C for 8 h, gene expression analysis was performed using RT-PCR and RT-qPCR. As a constitutive expressional control, RL32, a ribosomal gene, was used for expression analysis in RT-PCR and RT-qPCR. Each measurement was replicated three times with independent biological samples. Histogram bars annotated with asterisks represent significant ($P < 0.05$) difference between control and treatment in each tissue.

(B)

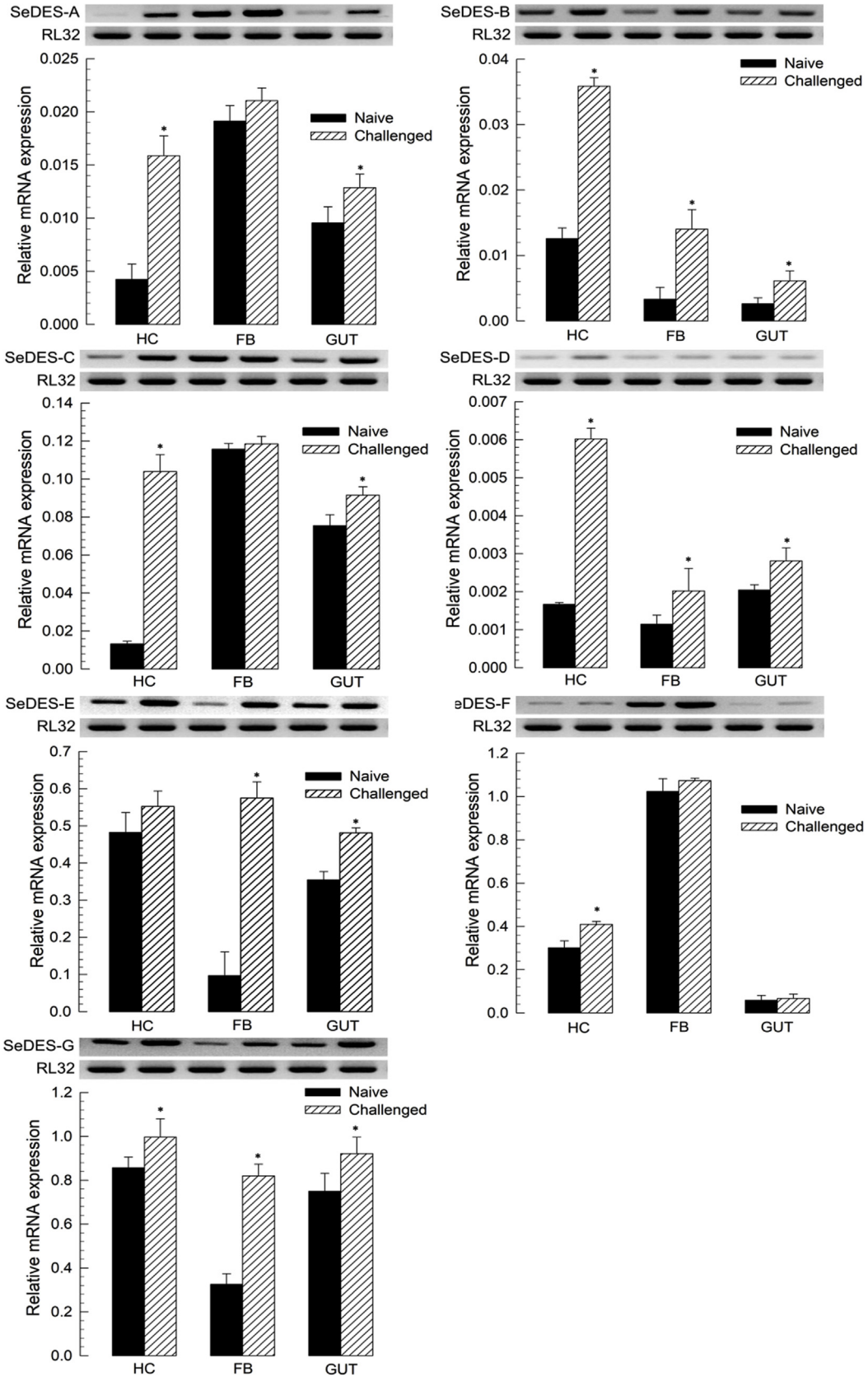
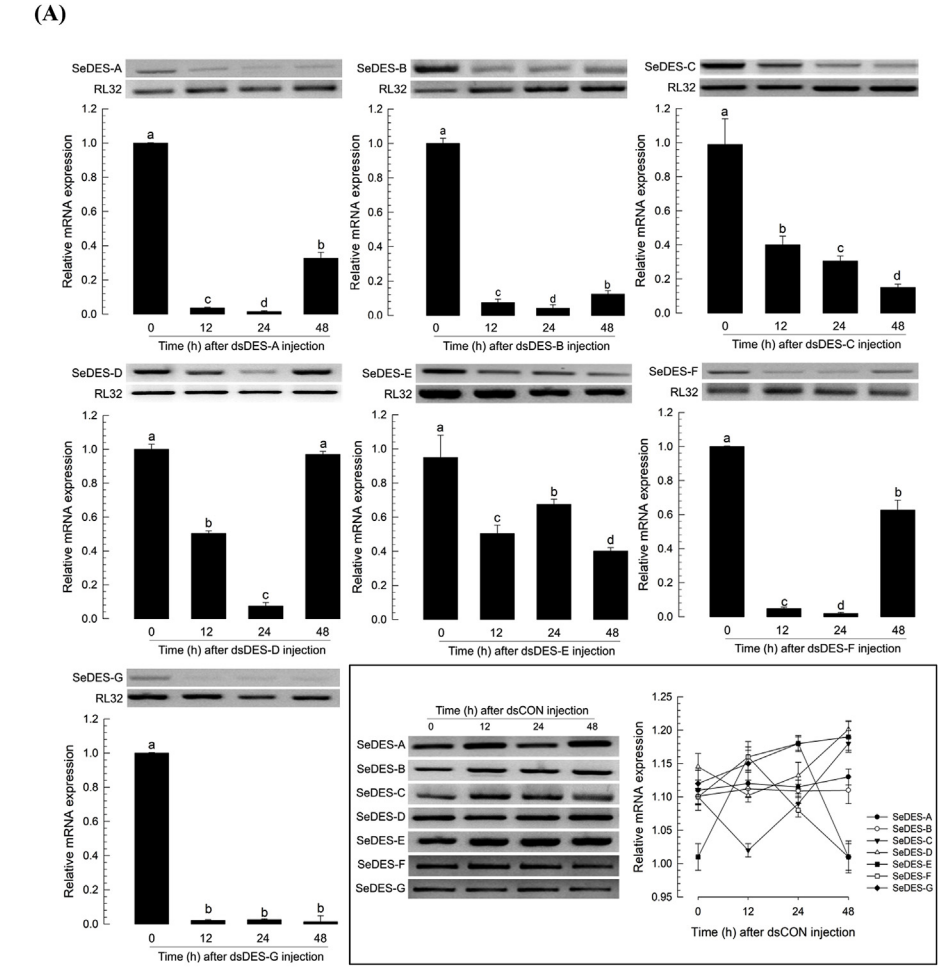


Fig. 5. (*continued*)



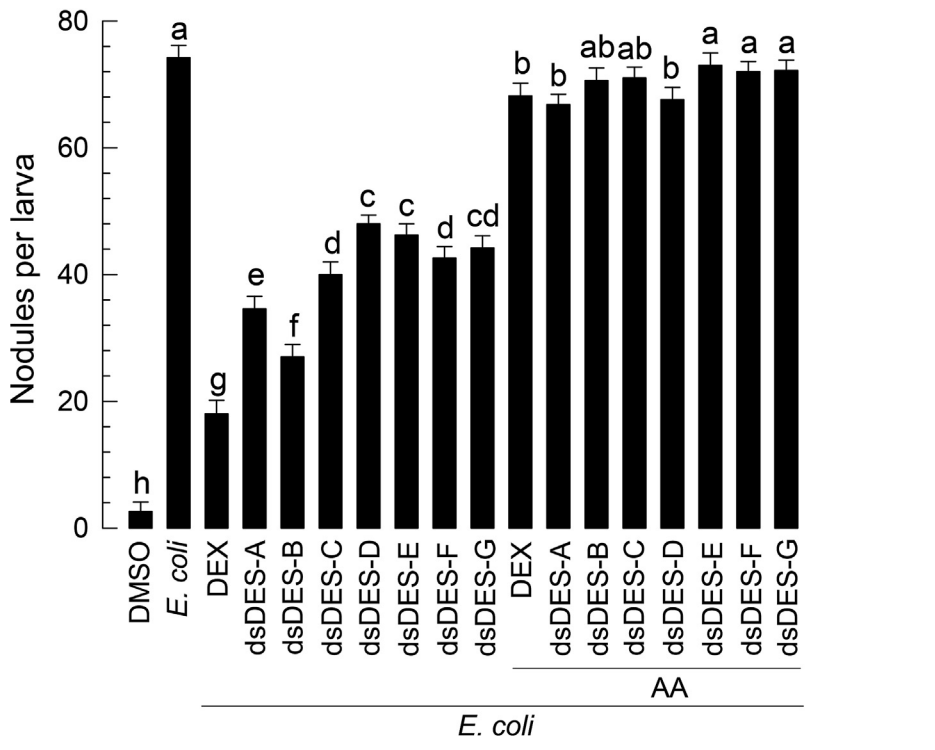


Fig. 6. RNA interference (RNAi) of *SeDESs* and subsequent functional assays for cellular immunity in *S. exigua*. (A) RNAi of *SeDES* expression with gene-specific dsRNAs (dsDES-A, dsDES-B, dsDES-C, dsDES-D, dsDES-E, dsDES-F, and dsDES-G). About 900 ng of dsDES was injected to each L5 larva. At 0, 12, 24, and 48 h PI, expression levels were assessed from whole body. As a constitutive expressional control, RL32, a ribosomal gene, was used for expression analysis in RT-PCR and RT-qPCR. As a control RNAi (dsCON), dsRNA specific to CpBV-ORF302 (a viral gene) was used for expression analysis. Each treatment was replicated three times. (B) Influence of SeDES RNAi on hemocyte nodule formation. At 24 h after RNAi, *E. coli* (1.8×10^5 cells/larva) was injected to L5 larvae. For rescue effect, AA (10 μg/larva) was co-injected with *E. coli*. After 8 h of incubation at 25 °C, treated insects were assessed for nodule formation. Histogram bars indicate nodules per larva and error bars indicate standard deviation. Histogram bars annotated with the same letter are not significantly different at Type I error = 0.05 (LSD test).

(A)

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FcEloV3-like    MEHICDTSAMSHVN-----LDSDPTYMKLYSF--EKFPKTWRIWGWCNWTVGIYASILYL-VILLVGIRLMKNRPPFRLQSALIGWNLGLSAFSVIFFSRSLPEIINAISSAGVLRSFCK  112
HmEloV6         -----MESPVMAYA-----YDITPNYSYIFEFENEFIHTDYRKWMTENWTVAFYYVGVYM-AFIFGGQYYMQNRPRFELRRTLMIWNCSLAAFSIMGACRTLPEFIHVLRNYGIYHSICV  109
SeEloV-D        -----MESPVMAYA-----YDITPNYSYIFEFENEFIHTDYRKWMTENWTVAFYYVGVYM-AFIFGGQYYMQNRPRFELRRTLMIWNCSLAAFSIMGACRTLPEFIHVLRNYGIYHSICV  109
VjEloV5-like    ------------------------MLLTRFLPARDPRTVQWFGAGDWITLLYILGMYTCTVKVLLPQYMANRKEYKLFGIIRLYNLLLVGVNAFFFCNLITRTY----LGGGYNWFCQ   90
PcEloV2-like    ------------------------------------------------------------------MRNRPAFDLKWFMVIYNLGLVALSLYMFLEVLLSVW----D-AEYDLLCS   45
HmEloV4-like    ------------------------------------------------------------------WLLVPYNLAMAGLNAYIALRLLTASF----R-LRYSYICE   35
SeEloV-C        ---------MADILINSTQSVLKETFDYYLWTLSLSDNRTKGWPLVDSPAPTVFYTLMYL-FIVWIGPKLMKNRRPFQLTWLLVPYNLAMAALNAYIAIRLLTASF----R-LRYSYYCE  105
SeEloV-B        ---METNNGISGIG-----DNSTEIRREP--VY-GNTDFVDSWFLMGSPIPVAIILASYLMFVLKIGPAFMMKRAPYQLSNVLLLYNAVQVAVSCFLFQKCFSMLV----ENGVVPKTCL  105
HsEloV1         -------------M-----EAVVNLYQE---VMKHADPRIQGYPLMGSPLLMTSILLTYVYFVLSLGPRIMANRKPFQLRGFMIVYNFSLVALSLYIVYEFLMSGW----L-STYTWRCD   94
BmEloV7         ---------MS-II-----SNVQRGFDYI--FDDLGDPRVKDWWLMKSPWPVLTVLGAYLYFCSYKGPQYMKDKKPYRLKNVLIVYNIVQIALSVYLTYTGLVYIY----K-TSYRFTCQ   98
SeEloV-A        ---------MS-VL-----ERMKDTYNYI--FYDLCDPRTRDWS--PSPIAIFTIIPFYLYFCNKLGPQLMKDEKPFKLKRIIMIYNIFQIIASGYIFYLGLFSGW----F-TTYSWTCQ   96
                                                                                                    I                  II
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FcEloV3-like    LE------NPSSAT-YFWGLIFCIFKIIEFGDTAFIVLRKQK--LIFLHWYHHLTVCIYGSFVWSTYDPAGRY-FYCMNAFVHAIMYFYYGLRAANMKPPKYLS--IVITSIQIMQMFVG  220
HmEloV6         PSFI----EQDKVS-GFWTWMFVLSKLPELGDTVFIVLRKKP--LIFLHWYHHITVLLYTWYSFTEYTSSARW-FVVMNYCVHSVMYSYYALVSMGKYPPKMLA--MTITVLQLTQMIVG  219
SeEloV-D        PSFI----EQDKVS-GFWTWMFVLSKLPELGDTVFIVLRKKP--LIFLHWYHHITVLLYTWYSFTEYTSSARW-FVVMNYCVHSVMYSYYALVSMGKYPPKMLA--MTIITVLQLTQMIVG  219
VjEloV5-like    GIGK----DRVDVAVLRLNYYYMFVRIFEFLDTVFFVLRKKFNQASVLNITHHCIAVGLPWYGLAYGLDGQICLMVLINMAVHVVMYSVYFLASF-SSIQPYLGWKRYLTLLQIVQFFVV  205
PcEloV2-like    VYNKNSWTNPKELRVANVLWWYFFSKAIEFMDTVLMVLRKKNEQITFLHVFHHASMLNIWWWTLMFIPGGMSWFGSCMNCLVHVVMYSVYGLSAL-PSLKGKLWWKRYITRFQLLQFCVT  164
HmEloV4-like    PCRQ--KYDPDELQIADAVWWYYFSKLLEFCDTFFFILRKKDEQLTFLHVYHHSTMFSFWWIGIKWVPSGSTFLPAMVNSGIHVLMYTYYGLSVFGPAVSKYLWWKKYLITMQLIQFTCA  153
SeEloV-C        PCRQ--KYDPDELQIADAVWWYYFSKLLEFCDTFFFILRKKEEQLTFLHVYHHSTMFSFWWIGIKWVPSGSTFLPAMVNSGIHVLMYTYYGLSVFGPAVSKYLWWKKCLTTMQLIQFTCA  223
SeEloV-B        ---M--ETDYSRRSITTAMYFYLLAKVSELLDTIFFILRKKNNQVTFLHVYHHAVMVFVTWTALKYEPTFALVFMGMLNSFIHIIMYTYYGLSAY-PNLVKYLWWKKYITRLQLLQFVLV  219
HsEloV1         PVDY--SNSPEALRMVRVAWLFLFSKFIELMDTVIFILRKKDGQVTFLHVFHHSVLPWSWWWGVKIAPGGMGSFHAMINSSVHVIMYLYYGLSAFGPVAQPYLWWKKHMTAIQLIQFVLV  212
BmEloV7         DINY--SNDPENLRWASSVVLYFIAKITELLDTVFFVLRKKQNQVTFLHLYHHTLMPLLSWYGTKYYAGGQAIPEGTVNSFIHVIMYGYYLVAGLGPQYQKYLWWKKHLTSMQLIQFCLV  216
SeEloV-A        PVDE--TDNPTTRTMHNAFWAYTIVKFIDMLDTVFFVLRKKDRQISFLHLFHHSMMPLASYIGLKYYPNGHATLLGLINSFVHVVMYTYVLIAGLGPKYQKYIWWKKHLTTMQLVQFATI  214
                                  III                IV                V                VI
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FcEloV3-like    IVISLFTSYVIL---TGGECDRPWSNIGV-ALAMYFSYCALFLNFFYQRYLRSAKKMKGEY-FDLTA---K------------KFVADKMF----------------------------  291
HmEloV6         CAINIWAHNYMAT-SAPTSCHISQINIKL-SMAMYFSYFVLFAQFFYKAYLSPKTGKKAKT-EPIPD---V------------RDVPVTKKVINGYPRQRNGVVAH------------  307
SeEloV-D        CAINVWAHNYMATTTPTSCHISQINIKL-SMAMYFSYFVLFAQFFYKAYLSPKTGKKAKT-EPIPD---V------------PVS-KKVNYNNGYPRQRNGVVAH------------  307
VjEloV5-like    VVHMS-------V-PIVHDCGYPVQNSIL-VIASYIYFLVMFAAFYDNAYQSRASKYATSKRLDSSHNGAARAHNDP-------LKIS-----------------------------  277
PcEloV2-like    FTHTV-------N-SIRIGCEFPRWGQNL-LSSYMVFMLFLFGNFYINAYIKRRSHRLKNGGAK------------------AKDICSSSSVANGNAKTSQASNLHRTKVE--------  248
HmEloV4-like    LVLGI-------N-GIRTGCEFPLWMHYV-LIIYMISFIILFGNFYMKAYIAKGSKCMEVRYGQ------------------CLD--DEETIANRKLKAN-----------------  224
SeEloV-C        LLLGI-------N-GIRTGCEFPLWMHYV-LIIYMISFIILFGNFYMKAYIAKGSKCMEVRYGQ------------------RLD--DEETIAKRKLKSN-----------------  294
SeEloV-B        LLQFG-------TSFATSSCPPSKIMFAS-MTFNVFLFIYLFGSFYINTYSKRNKKIEIAK--ESTGSNGIKVNELQKEDGEKGMRVCKDDKDYNRHLDNYTSTAIKLNDKYCFTFNLRT  329
HsEloV1         SLHIS-------QYYFMSSCNYQYPVIIHLIWMYGTIFFMLFSNFWYHSYTKGKRLPRALQQNGAPGIAKVKAN-----------------------------------------  279
BmEloV7         LYLNV-------VGLFSQNCNYPKSLNVI-VVTNCLAFLYMFGRFYYNSYVKNNRGKSEVEIKES-----------------------------------------------  273
SeEloV-A        FLHNL-------SALYENCGYPRWISTV-LCVHSLQFLYMFGNFYYVTYVKENKSNSNGSLKNG--SQKVKAH-----------------------------------------  277
                                                  VII
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FcEloV3-like    ----   291
HmEloV6         ----   307
SeEloV-D        ----   307
VjEloV5-like    ----   277
PcEloV2-like    ----   248
HmEloV4-like    ----   224
SeEloV-C        ----   294
SeEloV-B        KKLL   333
HsEloV1         ----   279
BmEloV7         ----   273
SeEloV-A        ----   277
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(B)

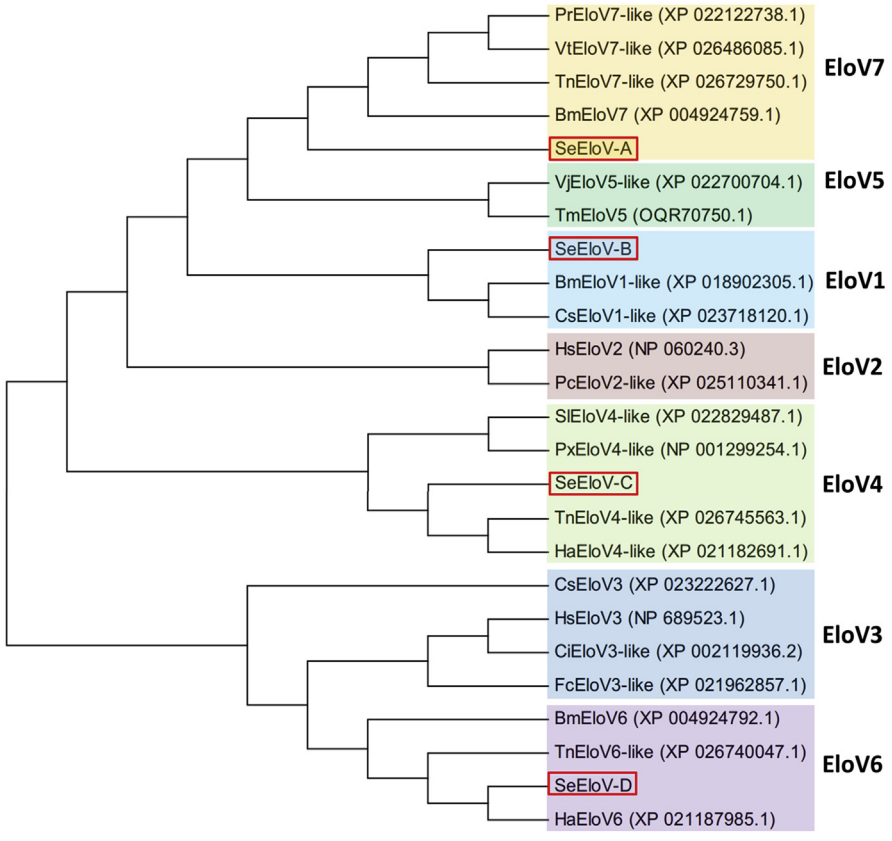

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Fig. 7. Prediction of very long fatty acid elongase (*SeEloV*) genes of *S. exigua*. (A) Sequence alignment and domain analysis of four *SeEloV* genes with other EloVs from invertebrates. Identical residues are marked by asterisks. Putative seven transmembrane domains (I ~ VII) are indicated with solid lines. Potential N-glycosylation sites are marked with black circles. Histidine boxes are framed in red color while other conserved motifs are shaded with blue color. Amino acid residues predicted for endoplasmic reticulum retention signaling are shaded with black color. Conserved domains were determined using InterPro tool (https://www.ebi.ac.uk/interpro/) and Prosite (http://prosite.expasy.org/). Other residues and motifs were predicted using tools from DTU bioinformatics (www.cbs.dtu.dk/services/). (B) Phylogenetic analysis of four SeEloV genes with other EloV types (EloV1 ~ EloV7) from other organisms. Amino acid sequences were retrieved from GenBank. Their accession numbers are provided in parentheses. The tree was constructed with Neighbor-joining method using MEGA6.0. Bootstrapping values on branches were obtained with 1,000 repetitions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

('HXXHH') (Fig. 7A). The alignment also revealed seven transmembrane-spanning regions with several potential N-glycosylation motifs along with an endoplasmic reticulum (ER) retention signal at the C-terminal region. These four SeEloV genes were clustered with different vertebrate and invertebrate EloV gene types (Fig. 7B). Phylogeny analysis suggested that these four *S. exigua* EloV genes were respectively classified into type 1 (*SeEloV-B*), type 4 (*SeEloV-C*), type 6 (*SeEloV-D*), and type 7 (*SeEloV-A*) elongases.

Although these four SeEloV genes were expressed in *S. exigua*, their expression levels showed significant ($P < 0.05$) variations among different developmental stages (Fig. 8A). Bacterial challenge up-regulated expression levels of SeEloV genes except for *SeEloV-D* in different larval tissues (Fig. 8B). Bacterial challenge significantly up-regulated all these three inducible SeEloV genes in the fat body. Immune challenge also increased expression levels of *SeEloV-A* and *SeEloV-B* in hemocytes.

RNAi suppressed expression levels of these SeEloV genes (Fig. 9A). In all four SeEloV genes, gene-specific dsRNAs significantly ($P < 0.05$) suppressed target mRNA levels at 12–48 h after injection. Under RNAi conditions, nodule formation mediated by AA was assessed (Fig. 9B). Only RNAi treatment against *SeEloV-B* suppressed nodule formation in response to bacterial challenge. AA addition to dsRNA treatment against *SeEloV-B* significantly ($P < 0.05$) rescued such suppressed immune response (Fig. 9C). These results suggest that *SeEloV-B* is associated with AA-mediating cellular immune response.

3.6. Exogenous AA injection reduces larval development and adult fecundity

Exogenous AA injection to naïve larvae of *S. exigua* induced an immune response by exhibiting nodule formation without bacterial challenge (Fig. 10). The number of nodules formed in response to AA injection was increased in a dose-dependent manner (Fig. 10A). Uncontrolled immune responses impaired larval development by reducing pupal weight (Fig. 10B) and adult fecundity (Fig. 10C). AA injection also induced expression of nine different AMP genes in three different larval tissues (Fig. S1).

4. Discussion

Eicosanoids play important roles in mediating various physiological processes in many terrestrial insects (Kim et al., 2018). However, biosynthetic pathways of eicosanoids are not clearly understood yet. Especially, terrestrial insects lack AA which is a main precursor for eicosanoid biosynthesis (Stanley, 2000). To understand eicosanoid biosynthesis in insects, AA biosynthetic pathways should be determined. This study proposed a biosynthetic pathway of AA and explored associated genetic factors in *S. exigua*.

Upon bacterial challenge, *S. exigua* larvae synthesized AA from fat body. Its level accounted for 0.2% of total fatty acids linked to phospholipids. Naïve larvae of *S. exigua* contained high amounts of LA (18:2n-6) in phospholipids of different tissues. Most animals are unable to synthesize LA. However, plants are known to synthesize LA (Stanley, 2006). In insects, most species are incapable of 18:2n-6 biosynthesis except for some species of Collembola, Blattodea, Orthoptera, Isoptera, Hemiptera, Coleoptera, and Hymenoptera (Stanley-Samuelson et al., 1994; Malcicka et al., 2018). Thus, *S. exigua* which is unable to synthesize C18 PUFA must obtain LA from diet because C18 PUFA is needed for *S. exigua* development. In the wax moth (*G. mellonella*) which is incapable of C18 PUFA biosynthesis, exogenous C18 PUFA is essential for adult metamorphosis (Dadd, 1983). On the other hand, lepidopteran insects are able to metabolize C18 PUFAs to C20 PUFAs as seen in the white butterfly (*P. brassicae*) in which larvae can develop to adults under 18:3n-3 supplementation. Subsequently, adults possess 20:3n-3 and 20:3n-3 (Parnanen and Turunen, 1987). Interestingly, in the current study, bacterial challenge induced the synthesis of C18 PUFA (γ-linolenic acid: GLA) and C20 PUFA (11,14-eicosadienoic acid: EDA) which might be other precursors in the ω-6 biosynthetic pathway of AA. This suggests that LA is desaturated at Δ6 to be GLA or elongated into EDA. This prediction was supported by results of rescue experiment against larvae treated with PLA_2 inhibitor to prevent a supply of eicosanoid precursor(s). Addition of AA significantly rescued immune responses. Similarly, LA, GLA, or EDA addition significantly rescued the immune response suppressed by PLA_2 inhibitor. However, α-linolenic acid (ALA) did not rescue the immunosuppression. These results suggest that AA can be synthesized from LA by chain extension and desaturase activities in *S. exigua*.

Long chain (C20-22) PUFAs (LC-PUFAs) have been identified as essential components of biological membranes of all cells. They play important roles in neural development and inflammatory response (van der Merwe et al., 2013; Awada et al., 2013). Vertebrates can biosynthesize LC-PUFAs such as AA, eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA) from C18 PUFAs of LA or ALA (Wymann and Schneiter, 2008). However, they cannot produce C18 PUFAs which should be supplied from diets. Two kinds of enzymes are involved in the production of LC-PUFA: desaturases (DESs) and elongase involved in very long chain fatty acids (EloVs). DESs are key enzymes that mediate the introduction of an unsaturation (double bond) into a fatty acyl chain while EloV catalyzes the condensation reaction within the elongation pathway and results in the addition of two carbons to fatty acid (Jakobsson et al., 2006; Guillou et al., 2010).

Nine different DES genes were annotated and their expression levels in *S. exigua* were assessed. These nine DESs encoded in *S. exigua* were classified into Δ5, Δ6, Δ9 DESs. All these genes appeared to be associated with AA biosynthesis because individual RNAi significantly prevented AA-mediated immune response. Specific inhibitor assays also supported the role of these three different types of DESs in inducing AA-mediated immune response. These results suggest that Δ9 DES would be responsible for production of unsaturated fatty acids while Δ5 and Δ6 DESs might be directly associated with AA biosynthesis by adding double bonds to specific locations. Based on our proposed model, Δ9 DES is not directly related with AA biosynthesis. However, its RNAi treatment impaired AA-associated immune response. This suggests that some of Δ9 DESs may catalyze desaturation at more than one site. Certain Δ9 DESs share structural similarity with Δ12 DESs especially in active site near histidine box (Malcicka et al., 2018), suggesting some Δ9 DESs of *S. exigua* behave like Δ12 DES to produce LA from oleic acid and enrich AA source. In addition, an unusual desaturase has been known in *S. exigua* that it catalyzes Δ11 and Δ12 desaturation reactions (Xia et al., 2019). Future research should focus on this scenario. Δ6-DES is responsible for the first and rate-limiting step in AA biosynthesis (see Fig. 1) where C18:2 is desaturated to GLA. The lack of Δ6-DES may activate a surrogate reaction in which C18:2 is elongated to EDA and

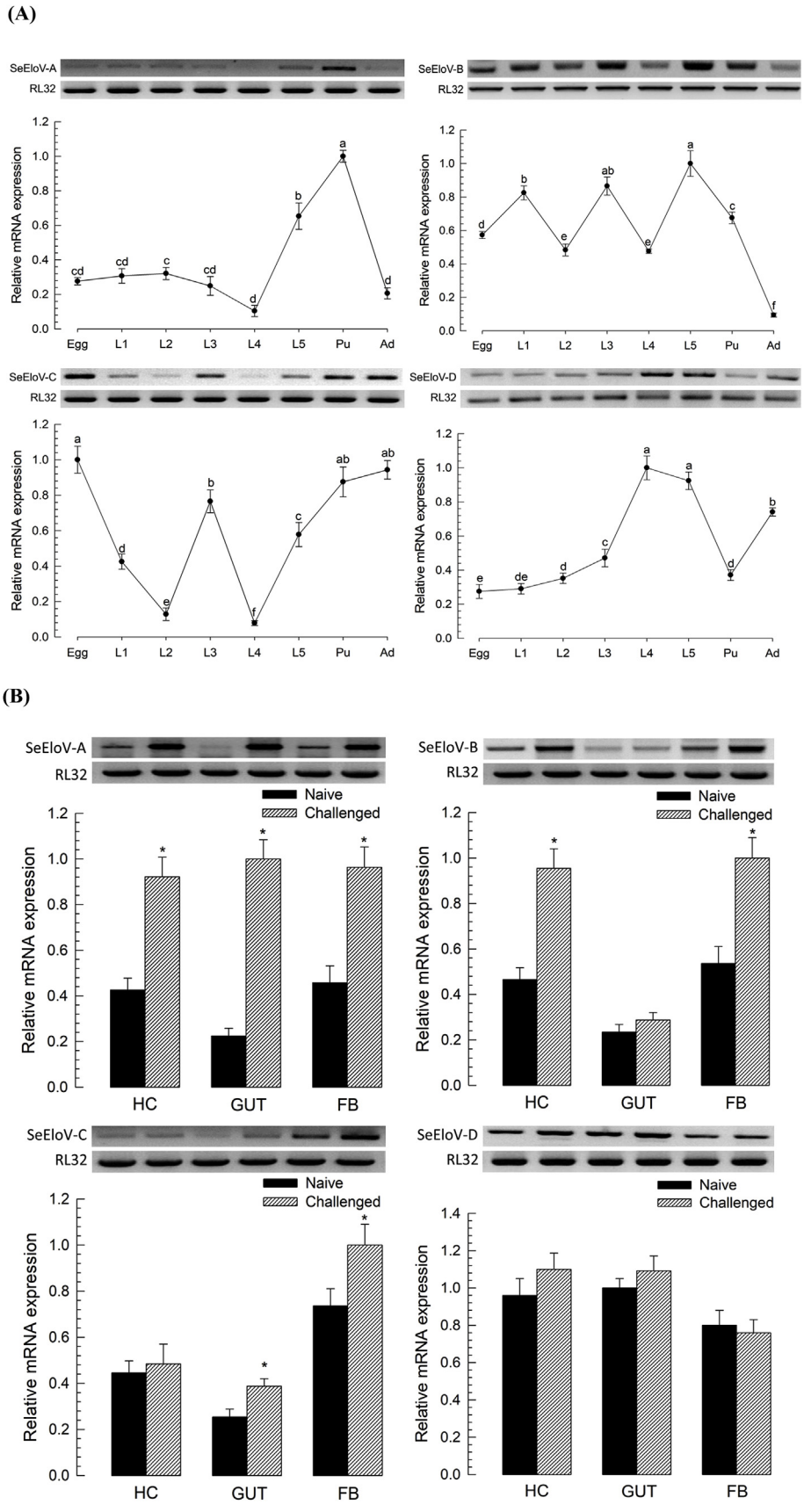

Fig. 8. Expression profiles of four *SeEloV* genes (*SeEloV-A* ~ *SeEloV-D*) in *S. exigua*. (A) Their expression patterns in different developmental stages [egg, larval instars ('L1-L5'), pupa, and adult]. Each measurement was replicated three times with independent biological samples. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test). (B) Influence of immune challenge on SeEloV expression in three different larval tissues [hemocyte ('HC), midgut ('GUT'), and fat body ('FB')]. Expression pattern was analyzed in naïve and *E. coli*-challenged insects. For immune challenge, individual L5 larva was injected with 1.8×10^5 cells of bacteria. After 8 h of incubation at 25 °C, gene expression was analyzed by RT-PCR and RT-qPCR. RL32, a ribosomal gene, was used as a constitutive expressional control for expression analysis in RT-PCR and RT-qPCR. Each measurement was triplicated independently. Histogram bars annotated with asterisks represent significant ($P < 0.05$) difference between control and treatment in each tissue.

then to eicosatrienoic acid (ETA: 8,11,14-20:3n-6). ETA is a precursor to produce 1-series prostaglandins (Stanley, 2000). Thus, the biological activity of EDA-mediating immune responses may be explained by the production of PGE1 or other 1-series PGs. Alternatively, the conversion of EDA to AA might be possible with catalytic activity of Δ8 DES. However, Δ8 DES was not detected from *S. exigua* transcriptomes

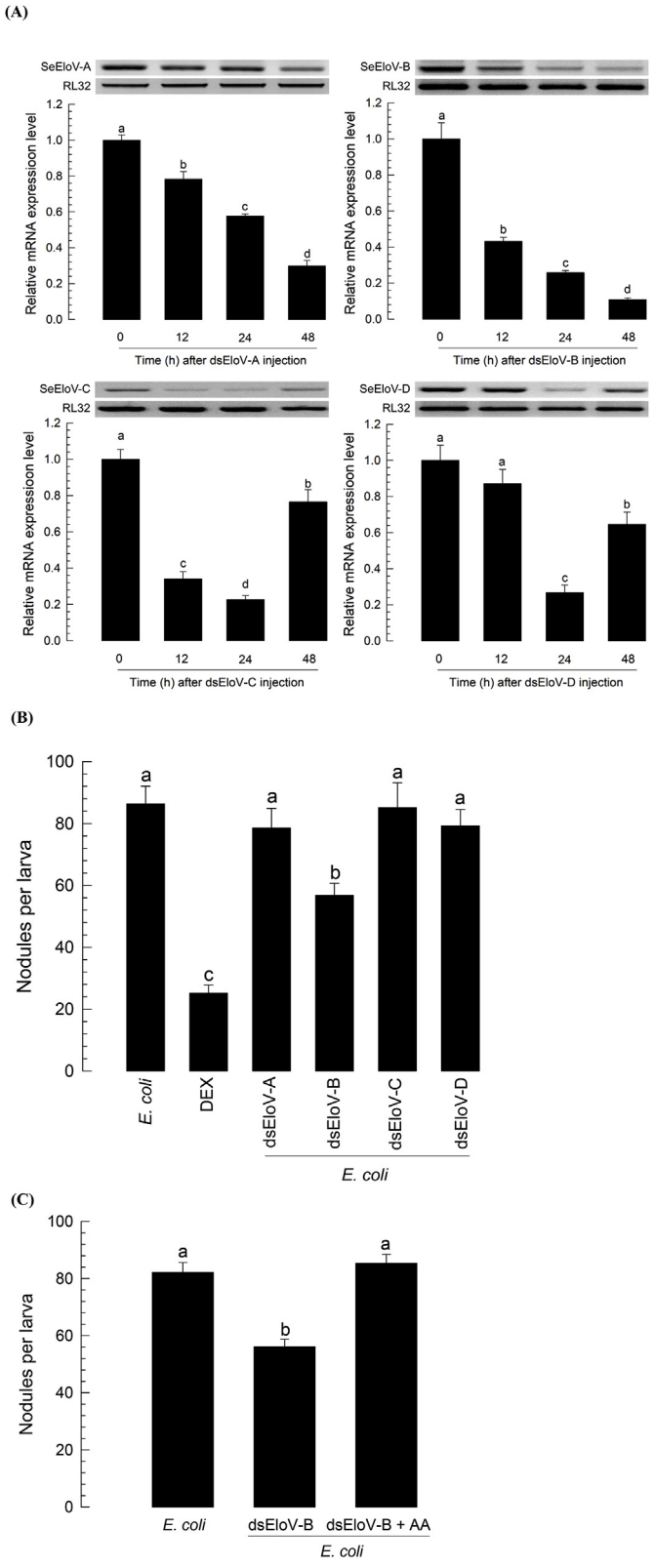

Fig. 9. RNA interference (RNAi) of *SeEloVs* and subsequent functional assays for cellular immunity of *S. exigua*. (A) RNAi of SeEloV expression with gene-specific dsRNAs (dsEloV-A, dsEloV-B, dsEloV-C, and dsEloV-D). About 900 ng of dsEloV was injected to each late L4 larva. At 0, 12, 24, and 48 h PI, expression levels of *SeEloVs* were assessed from whole insect. As a constitutive expressional control, RL32, a ribosomal gene, was used for expression analysis in RT-PCR and RT-qPCR. As a control RNAi (dsCON), dsRNA specific to CpBV-ORF302 (a viral gene) was used for expression analysis. Each treatment was replicated three times. (B) Influence of SeEloV RNAi on hemocyte nodule formation. At 24 h after RNAi, *E. coli* (1.8×10^5 cells/larva) was injected to L5 larvae. In separate experiments, AA (10 μg/larva) was co-injected with *E. coli* to determine rescue effect. After 8 h of incubation at 25 °C, treated insects were assessed for nodule formation. Histogram bars indicate nodules per larva and error bars indicate standard deviation. Histogram bars annotated with the same letter are not significantly different at Type I error = 0.05 (LSD test).

obtained from GenBank. Thus, its alternative pathway should be further explored in future study. In vertebrates, DGLA can be effectively converted to AA by Δ-5 DES because its knockdown by RNAi can prevent conversion from DGLA to AA (Xu et al., 2018). In our current assay, an addition of DGLA significantly stimulated the immune response mediated by AA in larvae treated with PLA_2 inhibitor. These results support that a biosynthetic pathway of AA in *S. exigua* follows a series of conversion from LA to AA via GLA and DGLA with catalytic activities of Δ-5 and Δ-6 DESs.

The proposed synthetic pathway needs catalytic activity of EloV to extend C18 PUFA to C20 PUFA. Elongases and ß ketoacyl-CoA synthase are endoplasmic reticulum enzymes responsible for the rate-limiting reaction of elongating PUFA carbon chain (Nugteren, 1965). At present, seven families of EloVs designated as EloV1-7 have been identified from mammals, with each family having a specific pattern of fatty acid substrate selectivity (Leonard et al., 2004). Among these families, EloV2 and EloV5 have received considerable attention due to their involvement in conversion of C18 PUFAs to C20 PUFAs (Naganuma et al., 2011). EloV4 is involved in elongation of very long-chain PUFA while EloV1, EloV3, and EloV6 mainly elongate saturated fatty acids and monounsaturated fatty acids (Tvrdik et al., 2000; Naganuma et al., 2011; Moon et al., 2014). In the current study, only one elongase encoded in SeEloV-B which was co-clustered with other EloV5 genes was associated with AA-mediated immune response. This suggests that AA is synthesized from LA with consecutive catalytic activities of elongase (SeEloV-B) and two desaturases (Δ5 and Δ6 DESs) in *S. exigua*. Although this study indicate that AA is biosynthesized from LA due to bacterial challenge it does not directly demonstrate the biochemical pathway. Future study should be performed to confirm the biosynthetic pathway by using stable isotope labeled LA and subsequent chemical analysis tracing the labeled compounds.

This study also showed that free and enough AA may have adverse effects on larval development and adult fecundity in *S. exigua*. Without bacterial challenge, AA injection alone induced cellular and humoral immune responses in *S. exigua*. This AA addition to larvae resulted in reduced pupal size and oocyte development in female adults. Excess eicosanoids derived from uncontrolled AA production might divert nutrient usage from development and reproduction to energy-consuming immune responses even without pathogen infection. This may result in fitness cost such as reduction in pupal body weight and adult fecundity. In honey bee, immune-challenge reduces expression and accumulation of stored plasma proteins such as vitellogenin and hexamerin while it highly induces immune-associated genes such as *defensin* and *prophenoloxidase*, suggesting an adaptive strategy to redirect resources to combat injury or infection (Lourenço et al., 2009). Indeed, immune challenge can mobilize stored nutrients in *Locusta migratoria* and bring about hyperlipaemia along with increase of lipophorins without mediation of adipokinetic hormone (Mullen et al., 2004). During hyperlipaemia, immune system is reconfigurated by suppressing surveillance function due to minimal free apolipophorin III to form low density lipophorins while increasing cellular immune responses by octopamine (Adamo, 2014). In *S. frugiperda*, PGs play crucial role in mediating cellular immune response and rescuing larvae infected with entomopathogen (Zhang et al., 2018). However, these rescued larvae suffered reduced survivorship compared to controls, indicating fitness cost as seen in the current study using *S. exigua*. This may explain the

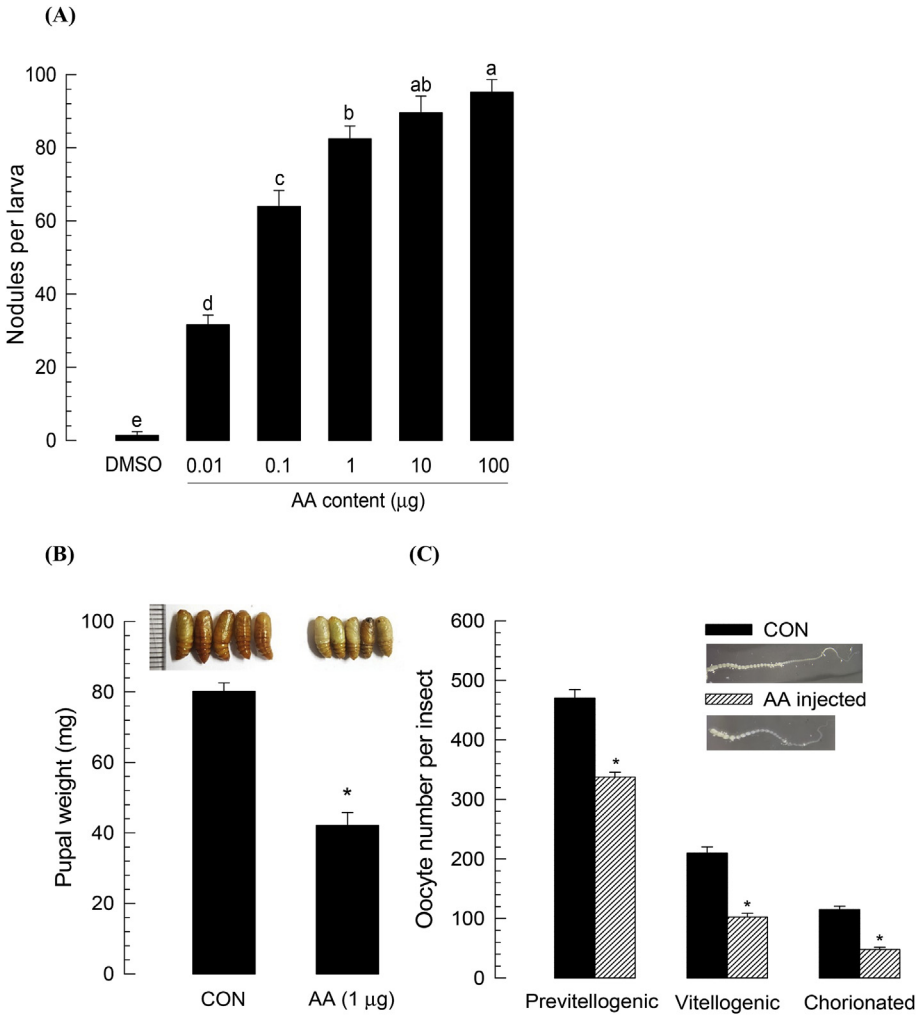

Fig. 10. Adverse effect of excess arachidonic acid (AA) on *S. exigua*. (A) Hemocyte nodule formation of L5 larvae upon injection of increasing amounts of AA. After 8 h of incubation at 25 °C, insects were checked for hemocyte nodule formation. Each measurement was replicated three times with independent biological samples. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test). (B) Influence of exogenous AA (1 μg/larva) on larval growth. AA was injected to L5 larva of *S. exigua* and incubated at 25 °C until pupation. (C) Influence of AA (1 μg/larva) on oocyte development of adult females. The experiment was triplicated with 10 insects in each replicate. Histogram bars annotated with asterisks represent significant ($P < 0.05$) difference between control and treatment.

reason why lepidopteran insects lack AA in lipid contents to prevent adverse effect induced by excess AA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103179.

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