Identification and expression of phospholipase A2 genes related to transcriptional control in the interleukin-17A/F1 pathway in the intestines of Japanese medaka *Oryzias latipes*

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ABSTRACT

Phospholipase A2 (PLA2), a phospholipid hydrolase, has recently attracted attention owing to its broad functionality. Immunological evidence has revealed increased susceptibility to infectious diseases and immunodeficiency in knockout (KO) mice of several pla2 genes. However, no progress has been made in terms of immunological research on any pla2 gene in fish. In this study, we focused on the intestinal immune responses of fish PLA2s. The full-length open reading frames of pla2g1b, pla2g3, pla2g10, pla2g12b1, pla2g12b2, and pla2g15 cDNAs were cloned in Japanese medaka (*Oryzias latipes*), and their gene expressions were quantified by real-time PCR (qPCR) and in situ hybridization (ISH). Characterization of pla2 genes revealed a functional domain and three-dimensional structure similar to the mammalian counterparts. In addition, expression of pla2g1b, pla2g12b1, and pla2g12b2 was extremely high in Japanese medaka intestines. ISH detected strong expression of pla2g1b mRNAs in the basal muscle layer, and pla2g12b1 and pla2g12b2 mRNAs were detected in the epithelial cells. In the medaka exposed to *Edwardsiella piscicida*, pla2g12b1, pla2g12b2 and pla2g15 were significantly induced in the anterior and posterior intestines, and pla2g1b was upregulated in the anterior intestine. Furthermore, pla2g1b, pla2g3, pla2g10, and pla2g12b2 were significantly downregulated in the IL-17A/F1 KO medaka compared to those in wild-type medaka. These results suggest that these PLA2s are involved in intestinal immunity in teleosts.

1. Introduction

Phospholipase is an enzyme that hydrolyzes ester bonds of glycerophospholipids; of these, phospholipases involved in the release of fatty acids are classified as phospholipase A (PLA). Furthermore, PLA is categorized into PLA1 and PLA2. PLA1 catalyzes the hydrolysis of the sn-1 position of glycerophospholipids, and PLA2 catalyzes the hydroxylation of the sn-2 position of glycerophospholipids [1]. By catalyzing the hydrolysis of the sn-2 position of glycerophospholipids by PLA2, stored arachidonic acid in the glycerophospholipid is released, which acts as the initiator for the synthesis of the lipid mediator that participates in various physiological activities in vivo [2].

Recently, the broad roles of PLA2 have received much attention because it has gradually been elucidated that PLA2 is also involved in other pathways in addition to the synthesis of lipid mediators. At present, more than 30 kinds of enzyme genes are reported to have PLA2-related activity [3]. Mammalian PLA2 is currently classified into groups 1–16 based on the properties of the amino acid sequences [4]. PLA2 is roughly divided into three groups based on the characteristics of its structure, properties, and tissue localization. PLA2 is roughly divided into cytosolic (cPLA2), Ca2+–independent PLA2 (iPLA2), and secreted PLA2 (sPLA2). Group 4 PLA2 (PLA2G4) corresponds to cPLA2, and PLA2G4 is further divided into six types (α, β, γ, δ, ε, and ζ) [5]. Except for cPLA2γ, cPLA2 has a C2 domain in the N-terminal region and a catalytic domain in the C-terminal region as the basic structure. cPLA2 depends on Ca2+ for its enzymatic activity, and in the presence of Ca2+, the binding between the C2 domain and cell membrane phospholipids is enhanced [6]. cPLA2 is currently found only in vertebrates, and its universal expression in various tissues has been confirmed [7]. It has also been suggested that cPLA2 is an important enzyme in the synthesis

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of lipid mediators in mammals [8,9]. iPLA2 is an enzyme that does not require Ca\(^{2+}\) for its activity and corresponds to group 6 PLA2 (PLA2G6). As it has a catalytic region similar to that of plant lipase patatin (iPLA2\(^{\alpha}\)), it is often called patatin-like phospholipase domain-containing (PNPLA). At present, nine types of PNPLA (1–9) have been identified, and they have a patatin domain in common. iPLA2 has been identified in a wide range of species, from plants to vertebrates, and is universally systemic in mammals [10]. In fact, in PNPLA8 KO mice, phenotypic abnormalities, such as decreased muscle mass, body temperature, and abnormal neurogenesis, due to abnormal lipid metabolism and mitochondrial dysfunction have been reported [11], which suggests that iPLA2 group is vital for basic life activities. sPLA2 is classified by classical taxonomy based on its similarity with sPLA2 contained in bees and snake venom, and a very diverse gene group is included in this sPLA2 group, which currently includes groups 1–3, 5, 9, and 13. Unlike cPLA2 and iPLA2, they are released extracellularly and show enzymatic activity in the presence of Ca\(^{2+}\). Because each cPLA2 has different tissue distribution and substrate selectivity, it plays a unique role in each organ [12,13]. In addition to the aforementioned three categories (cPLA2, iPLA2, and sPLA2), PLA2G7 and -8, corresponding to platelet-activating factor acetylhydrolase (PAF-AH) [14], and PLA2G15, corresponding to lysosomal phospholipase A2 (LPLA2) [15], have been reported.

Recently, several functional groups in the mammalian PLA2 family, particularly enzymes belonging to sPLA2, have been suggested to play important roles in the immune response of the host gastrointestinal (GI) tract, e.g., PLA2G1B, which is generally known as a digestive enzyme secreted by pancreatic gland cells into the lumen of the small intestine, and its substrate, a phospholipid contained in diet and bile. In addition to the digestive enzymes contained in a fluid secreted into the duodenum by the pancreas, cleavage of the N-terminal pro-peptide by trypsin is necessary for PLA2G1B activation in the lumen of the small intestine [16]. It has recently been reported that mammalian PLA2G1B secreted from intestinal epithelial cells is crucial for defense against parasitic infections. Typically, it is well-known that type 2 immunity is primarily involved in the defense response against parasitic infections in mammals. However, PLA2G1B KO mice cannot completely eliminate parasites, even during type 2 immune activation [17]. Furthermore, PLA2G2, which is known as sPLA2, is secreted by various types of cells such as hepatocytes, vascular smooth muscle cells, and endothelial cells, in humans and rats [18]. PLA2G2A expression in mice is localized in Paneth cells, which are known as antimicrobial peptide (AMP)-producing cells, in the small intestine and is used as a marker gene for the cells [18]. In mammals, the primary role of PLA2G2A is to protect against bacterial infection by degrading bacterial membrane phospholipids. In fact, it is known that interleukin (IL)-22 dependent STAT3 activation during Citrobacter rodentium infection strongly induces PLA2G2A production in the intestine [19]. In mammals, the function of PLA2 in the intestinal immune system is becoming increasingly clear.

Meanwhile, in teleosts, PLA2G1B has been confirmed to be expressed in the intestines of red sea bream (Pagrus major), annular seabream (Diplodus annularis), and Atlantic cod (Gadus morhua) [20–22] and in the gills of red sea bream (P. major) [23]. However, information about teleost PLA2s is rather limited, and an immune response in the intestine of sPLA2 genes, including PLA2G1B, has not been reported in teleosts. In our previous study, we showed that various lipid digestive enzyme, including pla2g1b, in the intestine of IL-17A/F1 KO medaka (O. latipes)
At present, of the various enzyme genes (F)<br/>(A)<br/>(pla2)<br/>(pla2g1b, pla2g3, pla2g10)<br/>(pla2g12b-1, (E) pla2g12b-2, and (F) pla2g15).

**Fig. 2.** Expression analysis of pla2 genes in various medaka tissues. The expression levels of these genes were determined by qPCR and are expressed relative to actb expression levels. Bars represent mean ± standard error (n = 3). (A) pla2g1b, (B) pla2g3, (C) pla2g10, (D) pla2g12b-1, (E) pla2g12b-2, and (F) pla2g15.

**Fig. 3.** The differences in the expression of pla2 family genes between the anterior and posterior intestines. The expression levels of these genes were determined by qPCR and are expressed relative to actb expression levels. Bars represent mean ± standard error (n = 3). *P < 0.05, **P < 0.01 (t-test).

were significantly downregulated compared to those in the wild-type (WT) [24]. The targeted teleost in our research, Japanese medaka (O. latipes) is a suitable model teleost because of its short life cycle and open genomic database [25]. At present, of the various enzyme genes downregulated in IL-17A/F1-KO medaka, we have primarily focused on pla2 genes and their importance in teleost intestinal immunity. In this study, we characterized six pla2s genes (five in sPLA2 group including: pla2g1b, pla2g3, pla2g10, and two pla2g12b; and one in LPLA2 group: pla2g15) in Japanese medaka. Furthermore, intestinal gene expression and response to E. piscicida infection of these genes were explored.

2. Material and methods

2.1. Animals

Japanese medaka were maintained in several transparent plastic tanks with a water circulating system (26 °C) under a 14-h light/10-h dark cycle. In all experiments, fish aged 3–4 months and weighing 200–300 mg were used. The Cab strain of Japanese medaka was used in all experiments, including molecular cloning of the pla2 open reading frame (ORF) nucleotide sequences, tissue distribution analysis, and bacterial infection tests. All animal experiments were conducted according to the relevant national and international guidelines and the “Act on Welfare and Management of Animals” (Ministry of the Environment, Japan). Ethics approval from the local IACUC was not sought as this law does not mandate the protection of fish.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from adult Japanese medaka using an RNeasy Plus kit (Takara, Shiga, Japan) according to the manufacturer’s instructions. Total RNA from each Japanese medaka was extracted, and the corresponding cDNA was synthesized separately (not normalized). Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was measured by the OD260/OD280 ratio with a minimum ratio of 1.8 used as a quality cut-off. cDNA synthesis was performed using 500 ng of total RNA extracted from each sample, and cDNA was prepared from the total RNA using ReverTraAce qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer’s protocol.

2.3. Cloning and sequencing

ORF nucleotide sequences of Japanese medaka pla2 mRNAs were identified from the medaka genome database in the Hd-rR strain (ASM223467v1) registered at NCBI (https://www.ncbi.nlm.nih.gov). The genetic order and orientation of pla2 genes were analyzed using the Ensembl genome database for comparison with other species in their syntenies. The specific primers for the medaka (Cab) pla2 mRNAs listed in Table S1 were designed to confirm the full-length ORF sequences. KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA), a high-fidelity PCR polymerase, was used for PCR. PCR products were cloned into the pTAC-2 vector (BioDynamics, Kumamoto, Japan). Plasmid DNA from at least three independent clones was purified using the Monarch DNA Gel Extraction Kit (NEB, UK), and sequencing analysis was performed using an Applied Biosystems 3730xl DNA analyzer (Foster City, CA, USA). Structural domains were predicted using a simple molecular architecture research tool (SMART version 7.0). The sequences generated were analyzed for similarity with other known sequences using FASTA and the basic local alignment search tool. cDNA sequences were directly compared using the gap program in BioEdit, and multiple sequence alignments were generated using ClustalW (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Domains and secondary structures were predicted using the SMART7 and PSIPRED software, respectively. Furthermore, SWISS-MODEL was used to predict the 3D structures. A phylogenetic chart was constructed using the full-length amino acid sequences for neighbour joining estimation using MEGA 7 (http://www.megasoftware.net).

2.4. Gene expression in different tissues

Various tissues, including the intestine, liver, spleen, gills, skin, muscle, brain, and kidney, were obtained from healthy Cab strain medaka (n = 3) to analyze PLA2 expression levels in each tissue using
real-time PCR (qPCR). In the intestine, the differences in expression between WT and IL-17A/F1 KO medaka were also confirmed. Total RNA was extracted from individual tissues, and their respective cDNAs were prepared as described in Section 2.2. Gene-specific primers were used to amplify the conserved regions of PLA2s. The medaka β-actin (actb) gene served as an internal control to confirm the quality and quantity of the cDNA used. The primer sets used are shown in Table S1. Relative expression ratios were calculated using the SYBR Green qRT-PCR method. SYBR green qRT-PCR amplification reactions were performed in duplicate in a total volume of 15 μL containing 7.5 μL of Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), 1.5 μL of cDNA, 1.5 μL each of forward and reverse primers (5 pmol), and 3 μL of distilled water. The qPCR program was run at 95°C for 15 s, 60°C for 30 s, followed by 40 cycles on a CFX connect TM (Bio-Rad, Hercules, CA, USA). A melting curve analysis of the amplified products was performed at the end of each cycle to confirm the specificity of amplification. The Ct value for the target amplification of each gene against the internal control were determined for each sample.

Using the comparative Ct method, the average Ct value from three determinations was used to calculate the relative expression levels using actb as the internal control.

2.5. In situ hybridization (ISH)

High expressions of pla2g1b and pla2g12b were observed in the intestine; therefore, we performed ISH to elucidate the intestinal localization of PLA2s. A gene-specific digoxigenin (DIG)-labeled RNA probe was synthesized using DIG RNA labeling Kit (SP6/T7) (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Gene-specific primer sets (Table S1) were designed to amplify full-length pla2 cDNAs for RNA probe synthesis. For sampling, the intestines were collected from healthy medaka, and the collected tissues were immediately fixed in 4% paraformaldehyde/0.1 M phosphate buffer overnight, embedded in paraffin, and sectioned at 8-μm thickness. For ISH, in brief, after dewaxing and rehydration, sections were permeabilized with proteinase K (Wako, Japan) in DEPC-treated PBS (final concentration, 5 μg/mL) at
37 °C for 15 min, re-fixed with 4% PFA/PBS for 10 min, and treated twice with DEPC-treated PBS containing glycine (2 mg/mL) for 10 min. Then, the sections were post-fixed with 4% paraformaldehyde/0.1 M PB for 5 min. Furthermore, prehybridization was performed for 2 h with a probe diluting solution [50% formamide, 5×SSC, 5×Denhardt’s solution (Wako, Japan) and 2 mg/mL RNA (Roche) in DEPC-treated water] after 30 min with 5×SSC/formamide. For hybridization, anti-sense RNA probes were diluted with a probe diluting solution (final concentration, 0.5 μg/mL), and hybridization was performed for 16 h at 55 °C. DIG detection was performed using HRP-labeled anti-DIG IgG antibody, and color was developed using NBT/BCIP solution (Roche, Basel, Switzerland).

2.6. Bacterial challenge

Japanese medaka were exposed by immersing in a tank containing E. piscicida (E381 strain) (26) at 7.4 × 10^7 CFU/mL. After immersion, the temperature of the tank was maintained at 28 °C and one-fifth of the water, including bacterial cells, was replaced every 24 h with fresh water. Medaka intestines were sampled separately at both the anterior and posterior sections, and three medakas were dissected at 12, 24, 48, and 72 h after bacterial challenge. Total RNA was extracted from all tissues, and cDNA was synthesized as described in Section 2.2. qPCR analyses were performed as described in Section 2.5.

2.7. Statistical analysis

All statistical calculations were performed using theR: A Language and Environment for Statistical (version 3.5.3) (27) in RStudio (version 1.2) (28). In the two-group comparison, an F-test was performed by var.

test to check the homogeneity of variance. Student’s t-test was used when homoscedasticity could be assumed between the two groups, and the Welch t-test was used when it could not be assumed. For multiple comparisons, the Tukey–Kramer test was performed without the F-test. In both cases, the significance was set at P < 0.05.

3. Results

3.1. Molecular cloning and sequencing

ORF sequences of pla2g1b, -g3, -g10, -g12b1, -g12b2, and -g15 from Japanese medaka of the Cab strain were determined, and these sequences were deposited in the GenBank database. The ORF of pla2g1b (Genbank accession no, LC599974), pla2g3 (LC599975), pla2g10 (LC599976), pla2g12b1 (LC599977), pla2g12b2 (LC599978), and pla2g15 (LC599979) were 450 bp, 921 bp, 465 bp, 645 bp, 861 bp, and 1260 bp, respectively, and could encode 149 amino acid (aa), 306 aa, 154 aa, 214 aa, 286 aa, and 419 aa, respectively. The theoretical molecular masses of were 16.96 kDa, 33.96 kDa, 17.33 kDa, 22.98 kDa, 31.40 kDa, and 47.68 kDa, respectively. In the pla2g3 mRNA of Japanese medaka Hd-rR, two splicing variants are presently deposited in Ensembl database [long form (565 aa), ENSORLT00000043434; short form (475 aa), ENSORLT0000005363]. In Cab medaka, the pla2g3 short form (921 bp) was cloned. However, the long form was not obtained by cloning with PCR amplification. Therefore, the identified sequence of the Cab strain pla2g3 was defined and deposited as a partial sequence deleted in the 5’-upstream region.
result, both the Ca-binding loop and His–Asp catalytic motif, which are important sites for enzyme activities and the characteristic structure of mammalian sPLA2, were observed to be conserved in PLA2G1B and -G10 of Japanese medaka of the Cab strain. Furthermore, these conserved proteins containing this domain include eukaryotic phospholipase A2 enzymes (PA2c) domains that enable the production of fatty acids and lysophospholipids by hydrolyzing the 2-ester bond of 1,2-diacyl-3-sn-phosphoglycerides. Additionally, Cab medaka PLA2G1B and PLA2G15, the transmembrane region and lecithin cholesterol acyltransferase domain, which promote the esterification reaction between cholesterol and phospholipid-derived acyl chains, were conserved, and the predicted 3D structure was also similar to that of mammals (Fig. S5).

3.3. Phylogenetic analysis

A phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap replicates. In the PLA2 family, analysis performed in all sPLA2 is presently characterized in mammalian species, viz., PLA2G1B, -G2, -G3, -G5, -G10, and -G12, in addition to lysosomal PLA2G15. As a result, teleosts PLA2G2 and -G5 have not been identified and deposited in the GenBank database. Medaka sPLA2 and PLA2G15, characterized in the present study, formed the same cluster with homologous genes in mammals and other fish species. PLA2G12B was divided into two clusters (i.e., PLA2G12B-1 and -G12B-2), with marine medaka and climbing perch also including two PLA2G12Bs, similar to Japanese medaka. Of these two fish PLA2G12Bs, the PLA2G12B-1 cluster was closer to mammalian PLA2G12B than to PLA2G12B-2 (Fig. 1).

3.4. Synteny

The medaka pla2 genomic organization was compared with those of other teleosts and mammals. In PLA2G1B of zebrafish and mammals, MS11 and COX6A1 genes were commonly located in the vicinity of PLA2G1B, but medaka msi1 and cox6a1 were located on different chromosomes from that of pla2g1b (Fig. S6). In pla2g3, medaka and zebrafish had similar synteny, but these were different from those of mammals. In contrast, RNF185, which is tandemly located in the mammalian PLA2G3, was encoded on the same chromosome in the case of medaka pla2g3 (Fig. S7). In PLA2G10 and PLA2G15, no similar synteny was found between teleosts and mammals (Figs. S8, S9, S10). Regarding PLA2G12B, Japanese medaka pla2g12b2 showed a similar synteny to those of mammalian PLA2G12B (Fig. S9).

3.5. Gene expression in intestine and other tissues

qPCR analyses of all medaka tissues revealed that each pla2 showed different expression patterns between genes and organs. In pla2g1b, pla2g12b1, and pla2g12b2, high expression was detected in the hepatopancreas and intestine (Fig. 2A, 2D, 2E). Furthermore, high expression of pla2g3 in the brain and that of pla2g10 in skin was observed (Fig. 2B, 2C). However, pla2g15, which is a lysosomal enzyme, was ubiquitously expressed in all organic tissues sampled (Fig. 2F).

Furthermore, regarding the intestine, sampling was performed separately for the anterior and posterior sections, and expression level of each gene was quantified by qPCR. As a result, the expression of pla2g3, pla2g12b1, and pla2g12b2 in anterior intestine were significantly higher than that of posterior intestine (Fig. 3). Furthermore, regarding pla2g1b, pla2g12b1, and pla2g12b2, which showed high expressions in the intestine, the localization of mRNAs was histologically clarified by ISH. As a result, pla2g1b was observed to be strongly expressed in the muscle layer at the base of the intestinal tract, and pla2g12b1 and pla2g12b2 were observed to be expressed in the intestinal epithelial cells (Fig. 4) with no signal in negative control using each sense probe for hybridization (Fig. S11).

3.6. Bacterial challenge

Bacterial exposure by immersion in E. piscicida-containing water tank was performed, and expression changes of each PLA2 gene in the anterior and posterior intestines were quantified by qPCR. As a result, in the anterior intestine, pla2g1b, pla2g12b1, and pla2g12b2 increased.

Fig. 6. The comparison of gene expressions of six pla2 genes between WT and IL-17A/F1 KO medaka intestine. Whole intestinal tissues were sampled and total RNAs were extracted for comparison (n = 4). The expression levels of these genes were determined by qPCR and are expressed relative to actb expression levels. Bars represent mean ± standard error. *P < 0.05, **P < 0.01 (t-test).
significantly 12 h (h) after E. piscicida exposure (Fig. 5A, 5D, 5E). pla2g15 in anterior intestine also increased significantly from 12 to 48 h (Fig. 5F). Conversely, pla2g93 decreased significantly after 12-48 h (Fig. 5B). In pla2g10, no significant changes were observed in the anterior intestine (Fig. 5C).

In the posterior intestine, no significant changes were observed in the expression of pla2g1h, pla2g3h, and pla2g10. However, pla2g2b1 and pla2g2b2 increased significantly after 72 h (Fig. 5J, 5K). Furthermore, the expression level of pla2g15 also increased after 72 h (Fig. 5L).

3.7. Intestinal expression of PLA2 genes in IL-17A/F1 KO medaka

In the whole intestine of IL-17A/F1-KO medaka, pla2g1h, pla2g3h, pla2g10, and pla2g2b2 genes were significantly downregulated compared to those in WT medaka. However, the secretory PLA2 pla2g2b1, which is predominantly expressed in the intestine, showed no significant change in both KO and WT medakas as well as lysosomal PLA2, pla2g15 (Fig. 6).

4. Discussion

To date, little progress has been made in research on pla2 genes in teleosts. In our previous study, Japanese medaka pla2g1b was identified as a downregulated gene in the RNA-Seq results of IL-17A/F1 KO medaka intestines [24]. To the best our knowledge, this is the first study to report the identification of other medaka pla2 genes, including pla2g3h, pla2g10, pla2g2b1, pla2g2b2, and pla2g15, at the genetic level in teleosts. Moreover, tissue distribution of the six pla2 mRNAs and their responses against pathological exposure in the anterior and posterior intestines were elucidated. Phylogenetic analysis showed that all types of medaka sPLA2 (including PLA2G1B, -3, -10, -12B-1, -12B-2, and -15) formed the same clusters as PLA2 in mammals. However, the pla2g2 gene homologue in Japanese medaka and other fish species could not be found in the GenBank database. Among them, PLA2G2A in mice is an antimicrobial molecule secreted by Paneth cells, which are AMP-producing cells in the intestinal epithelium, and is used as a typical marker for Paneth cells in mice. However, in teleosts, pla2g2, including pla2g2a, has not yet been identified. In addition, previous histological studies in teleosts showed no Paneth cells in the fish intestine [29].

Gene expression analysis in various tissues revealed that the distribution of six medaka pla2 mRNAs [five pla2 genes (sPLA2 group): pla2g1h, pla2g3h, pla2g10, two pla2g12hs; and one pla2 (LPLA2 group): pla2g15] was quite different and localized among all organs. pla2g1h, pla2g2b1, and pla2g2b2 in the hepatopancreas and intestine; pla2g3h in the brain; and pla2g10 in the skin showed extremely high expressions among all tissues examined. Similar to our results in Japanese medaka, the expression of each sPLA2 in mammals also showed a biased distribution among all organs. However, the tissue localization patterns of sPLA2 mRNAs do not always exhibit consensus pattern among mammalian species. Human PLA2GB1 is highly expressed in the hepatopancreas [30], but mouse PLA2GB1 is expressed in the stomach and intestines [31]. PLA2GB2 expression is high in the hepatopancreas and small intestine in both humans and mice [30,31]. In addition, PLA2G3 is highly expressed in human skin [30], mouse colon [31], and rat brain [32], which is consistent with the results of the medaka pla2g3 in the present study. The expression level of PLA2G10 is high in the stomach and intestine of humans and rodents [30-32]. However, medaka pla2g10 was remarkably high in the muscle and epidermis, unlike the expression pattern in mammals. However, no previous reports have described teleost pla2g10. In addition, mammalian PLA2G15 is ubiquitously expressed in all tissues examined [30-32], similar to medaka pla2g15. Furthermore, the expression of Japanese medaka pla2g3, pla2g12b1, and pla2g12b2 were significantly higher in the anterior intestine than in the posterior intestine. The distribution of the expression was similar to that observed in mouse PLA2G3 in the small intestine and colon [31]. Thus, the tissue distribution patterns of Japanese medaka pla2 mRNAs are mostly conserved in mammals.

In addition, regarding pla2g1b and two pla2g12b mRNAs, which were highly expressed in the medaka intestines, their histological localizations in the anterior and posterior intestines were clarified by ISH. pla2g1b signal was detected in the muscle layer, and two pla2g12b signals were detected in intestinal epithelial cells. In the mammalian small intestine, PLA2G1B is locally expressed in a part of epithelial cells [17]. Meanwhile, strong expression of red seabream pla2g1b has been observed through the entire intestinal tissue, including muscle layer and epithelial cells, by ISH [20]. However, pla2g12b has not been studied in fish so far. In mammals, although it has been clarified that the gene expression level itself is extremely high in the intestinal tract, no previous reports have described the histological localization at the mRNA or protein levels in the intestines. Therefore, it is a very interesting and novel insight that both pla2g12b1 and pla2g12b2 were expressed in the intestinal epithelial cells in the present study. Although PLA2G12B has been identified as a new sPLA2 family member in mammals, research on its physiological properties has hardly progressed. This stagnation of the progress may be due to the fact that PLA2G12B does not harbour the His-Asp catalytic motif, which is conserved in other sPLA2s, and is thought to have no activity as a PLA2 enzyme [33]. However, in recent years, limited data on the function of PLA2G12B have been reported in mammals. In PLA2G12B KO mice, the secretion of very-low-density lipoprotein (VLDL)-triglycerides (TG) synthesized in the liver was significantly inhibited, and these over-stored were also observed in hepatocytes, suggesting the role of the gene in lipid metabolism, particularly in lipid transportation [34]. Furthermore, hepatitis C virus (HCV) infection upregulates PLA2G12B at the transcriptomic level via the activation of nuclear transcription factor, hepatocyte nuclear factor 4α (HNF4α), activation, and the increase in PLA2G12B seems to facilitate mature HCV secretion as well as the mechanism of host VLDL secretion [35]. However, no studies on the response against bacterial infection have been reported in both mammals and teleosts, other than our results. In the present study, the expression of both pla2g12b1 and -2 was significantly induced in the posterior intestine of Japanese medaka after exposure to E. piscicida. These results suggest the involvement of fish PLA2G12B in intestinal immunity, particularly in the posterior intestine. In the posterior intestine of zebrafish, neutrophil density and the expression of inflammatory cytokines, including interleukin (IL)-1β, IL-8 (ββ), and IL-17 (ααα), and AMP, including β-defensin (defb), C-type lysozyme (lyz), and other immune-related genes, have been reported to be highly expressed in the posterior intestine than in the anterior intestine [36]. In mammals, the expression of Pla2g2 mRNA was significantly impaired in IL-17F KO mouse colon but not in that of IL-17A KO mouse, relative to such expression in WT colon, and the expression of Pla2g2 was also enhanced only by the recombinant IL-17F (rIL-17F) in IL-17F KO colonic cells [37]. Additionally, the expression of PLA2G10 was directly regulated by the rIL-17A stimulation in primary airway epithelial cell cultures of mice [38]. However, the mechanism of sPLA2 regulation through IL-17 pathway is still unknown. In our present study, the downregulation of four pla2 genes, viz., pla2g1b, pla2g3h, pla2g10, and pla2g12b2, in the 117A/F1 KO medaka suggests some relationship to the sPLA2 transcriptional regulation in the IL-17 pathway.

Other than pla2g12b, the expression of pla2g1h in the anterior intestine and pla2g15 in both sectional intestines was significantly increased by E. piscicida exposure. The general role of mammalian PLA2G15 is the degradation of dietary phospholipids. However, it is also known to be vital in defending against parasitic infections [17]. PLA2G15, an important lysosomal enzyme, degrades phospholipids in up-taking antigens. In fact, PLA2G15 KO mice have been reported to inhibit antigen presentation to CD1d and hypoactivity of immune responses against microbial lipids and auto lipid antigens [39]. In the future, it will be necessary to analyze anti-microbial activities and establish mutant medaka lines to elucidate the role of fish PLA2s in intestinal immunity against bacterial exposure. In particular, we expected
that the functional analysis of PLA2G12B will provide novel and interesting insights into the mechanisms of defense against bacterial pathogens in fish intestines.

In conclusion, we characterized Japanese medaka pla2g1b, pla2g3, pla2g10, pla2g12b1, pla2g12b2, and pla2g15 and performed gene expression analyses, particularly in the intestines. Japanese medaka PLAs showed relatively high sequence similarity in amino acid levels with those of mammals. In expression analysis, pla2g1b, pla2g12b1, and pla2g12b2 showed high expression level in the intestines, and histological localization was confirmed by ISH. In medaka exposed to *E. piscicida*, pla2g12b1 and pla2g15 genes were significantly induced in the anterior and posterior intestines and pla2g1b was upregulated in the anterior intestine. Furthermore, pla2g1b, pla2g3, pla2g10, and pla2g12b2 genes were significantly downregulated in the IL-17A/F1 KO medaka compared to those in WT medaka. These results suggest that these PLAs are involved in intestinal immunity in fish similar to that in mammals.

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**Declaration of Competing Interest**

None.

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**Supplementary materials**

Supplementary material associated with this article can be found, at the online version, at doi:10.1016/j.fsi.2021.100028.

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