EXPRESSION OF GROWTH HORMONE (*Ph*GH) GENE AND ANALYSIS OF INSULINE-LIKE GROWTH FACTOR I (IGF-I) PRODUCTION IN AFRICAN CATFISH (*Clarias gariepinus*) TRANSGENIC F-1

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ABSTRACT

We have previously produced F-1 transgenic of African catfish from crosses between founder transgenic female and non transgenic male. The aim of this study was to evaluate distribution and expression *Ph*GH growth hormone gene transgenic African catfish organs and to measure the concentration of IGF-I in plasma. Transgene was detected using the PCR method in various organs, namely pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, gill, and eye. Transgene expression levels were analyzed using the method of quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR). Plasma samples were analyzed for Insuline-like Growth Factor (IGF-I) using Enzyme Linked Immunosorbent Assay (ELISA) method. The results showed that the *Ph*GH was detected and expressed in all organs of the transgenic African catfish (F-1). Liver exhibited the highest level of *Ph*GH mRNA (23 x 10^6 copies). The plasma IGF-I levels in transgenic individuals were not significant than non transgenic. The higher level of exogenous *Ph*GH gene expression may not represent the production of IGF-1.

KEYWORDS: gene expression, transgenic, growth hormone, *Clarias gariepinus*, RT-PCR, IGF-1

INTRODUCTION

Genetically modified fish have also been developed as experimental models for fish breeding research, specially in studies increasing aquaculture productivity using transgenesis. Transgenic fish are of value both as model species in fundamental research and as potentially genetically superior brood-stock for commercial food production (Hew *et al.*, 1995; Iyengar *et al.*, 1996).

Now days, growth hormone (GH) gene transgenesis has also been used in several fish species to enhance growth. Du *et al.* (1992) have demonstrated that, following introduction of a DNA construct (OPAFPcsGH), in which an ocean pout (*Macrozoarces americanus*) antifreeze promoter (AFP) drives a chinook salmon (*Oncorhynchus tschawytscha*) GH cDNA sequence, transgenic Atlantic salmon (*Salmo salar*) exhibited impressive growth enhancement. Later, Devlin *et al.* (1994); Mori & Devlin (1999) observed a dramatic improvement in growth of transgenic coho salmon (*Oncorhynchus kisutch*) by the expression of a sockeye salmon (*Oncorhynchus nerka*) GH gene driven by a metallothionein promoter (MT-B) of the same species. Nam *et al.* (2001) have also demonstrated dramatic acceleration of growth in transgenic mud loach (*Misgurnus mizolepis*) after microinjecting the mud loach GH gene fused to the mud loach β-actin promoter. Other commercially significant species in which transgenic growth enhancement has been attempted include channel catfish (Dunham *et al.*, 1987), rainbow trout (Chourrout *et*

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al., 1986; Penman *et al.*, 1990), common carp (Zhang *et al.*, 1990; Rosochacki *et al.*, 1993) and northern pike (Gross *et al.*, 1992; Guise *et al.*, 1992). Although probable integration of the transgenes into the genome was demonstrated in all of these research. However, GH exogenous gene expression may not in all organ in transgenic fish. The different expression was sometimes attributed to the use of promoter sequences from very distantly related species.

The exogenous gene expression may effects through direct or indirect by inducing insulin-like growth factor I (IGF-I) release from the liver into the bloodstream or the local production of IGF-I in different organs. Butler & Leroith (2001) reported that insulin-like growth factor I (IGF-I) is a potent mitogenic hormone that induces growth and differentiation in a variety of target organs. The principal hormones in the GH/IGF axis are pituitary GH and liver-derived endocrine insulinlike growth factor-I (IGF-I). IGF-I circulates bound to a number of IGF-binding proteins (IGFBPs), which modulate its growth-stimulatory effects (Pierce *et al.*, 2005). The aim of this research is to evaluate distribution and expression *Ph*GH growth hormone gene transgenic African catfish organs and to measure the concentration of IGF-I in plasma by ELISA.

MATERIALS AND METHOD

Animal and Sample Collection

In this study, F_1 transgenic African catfish (4 month of age) carrying the pCcBA-*Ph*GH construct were generated by electroporation with 100 µg/mL of the construct (Dewi *et al.*, 2011). All fish were derived from a single family of African catfish (*Clarias gariepinus*) and originated from the collection in Research Institute for Fish Breeding, Indonesia.

Detection and Distribution *PhGH* in Transgenic African Catfish F-1

DNA Extraction

*Ph*GH gene detection performed organs. Genomic DNA of each sample was extracted using DNA extraction kit following the protocols recommended by manufacturer (GeneJet Genomic DNA Purification, Thermo Scientific). Briefly, the protocol consists of series of steps including Cell lysis, DNA precipitation, binding DNA to the column, washing, and elution. Cell lysis was performed by weighing of approximately 10 mg of tissue sample and mincing it with a surgical blade into small pieces. The tissue was lysed by adding 180 µL of lysis digestion buffer, 20 µL of proteinase K, followed by incubation in 56°C for 2 hours and adding 20 µL of RNase A solution followed by incubation in room temperature for 10 min. The lysate was added 200 µL with lysis solution followed by vortexing for 15 second. The lysate DNA was then precipitated by adding 400 µL of 50% ethanol. Binding DNA to the column was carried out by spinning the mixture lysate DNA and ethanol at 6,000 x g for 1 min. The column binding DNA was then washed twice by adding 500 µL of washing buffer I and washing buffer 2 followed by spinning at 8,000 x g for 3 min. and 12.000 x g for 3 min., respectively. Finally, the DNA bound to the column was then eluted by adding 200 µL elution buffer, incubating it for 2 min. in room temperature and terminated by spinning it at 8,000 x g for 1 min. To check the success of genomic DNA extraction process, the sample was run on mini horizontal gel electrophoresis. The sample was loaded in to the 1.5% (w/v) agarose gel, powered with 65 volt electricity and run for 50 minutes. The gel was then stained with ethidium bromide (0.5 µg/mL) and viewed using gel documentation system ultraviolet transillumination.

Polymerase Chain Reaction (PCR)

Amplification of *Ph*GH was performed using thermal cycling system (mycycler, Biorad). PCR standard was performed in a final volume of 25 µL, using a comersial kit master mix faststart PCR master (Roche, Germany). The PCR was used to amplify a 392-bp fragment with primers *PhGH*1-F (5'- TAG AGT GTT GGT GGT GCT CTC TGT -3') and *PhGH*2-R (5'- CGA TAA GCA CGC CGA TGC CCA TTT-3'), for 35 cycles: denaturing at 94°C for 30 secs, annealing at 55°C for 30 secs, and extension at 72°C for 1 min. The amplification using the specific primer β-actin for internal control: bact-F (5'-TAT GAA GGT TAT GCT CTG CCC-3') and bact-R (5'-CAT ACC CAG GAA AGA TGG CTG-3') were designed from a catfish growth hormone sequence. The reaction was incubated at 94°C for 30 secs, annealing at 55°C for 30 secs, and extension at 72°C for 1 min. for 30 cycle, and the PCR products separated on 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 µg/mL) and visualized by ultraviolet transillumination.

Analysis Expression of *PhGH*

RNA Extraction

Total RNA was extracted from pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, thymus, gill, and eye from three transgenic and three non-transgenic fish using animal RNA extraction kit following the protocols recommended by manufacturer (Tri Reagent Molecular Research Center, Inc., Cincinnati, OH, USA). Briefly, a small amount of fresh tissue was homogenized in Tri-Reagent RNA (1 mL per 50-100 mg tissue) followed by incubation for 5 mins at room temperature. After added 100 µL bromochloropropane per 1 mL Tri Reagent followed by shake 15 secs, incubated for 15 mins at room temperature and spinning 12,000 x g for 15 mins at 4°C. The aqueous phase was collected and precipitated with 0.4 volumes of isopropanol, and stored at 4°C for 5 min. The precipitated RNA was washed with 1 mL 75% ethanol and resuspended in 200 µL RNAsecure (Ambion) and heated at 60°C for 10 mins. Total RNA was diluted to 1.0 mg/mL in RNase-free water and photospectrometrically quantified at A_{260}. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). For synthesizing 1st-strand cDNA, 50 µg of total RNA was treated with commersial kit cDNA Ready-To-Go™ RT-PCR Beads (GE Healthcare) according to the protocol suggested by the manufacturer and 1 Oligo d $(T)_{16}$ primer (Roche, Germany) in a final volume of 33 µL. cDNA concentration was always determined with gene Quant (Qiagen), which was more accurate than spectrophotometric determination (OD260/280).

Real-Time PCR Analysis

The RT-PCR was formed to detect *Ph*GH gene in 25 embryos pooling, 25 larvae pooling and pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, gill, thymus, and eye organs. The qPCR was conducted with 100 ng DNA in duplicate in a 25 µL reaction. Real-time PCR was performed using Rotor-Gene SYBR Green kit (Qiagen) and the Rotor Gene Real-Time PCR System (Qiagen), with the following parameters: 95°C for 10 sec., followed by 40 two steps cycles at 95°C for 5 sec. and at 60°C for 30 sec. Primers for the *PhGH* gene were *PhGH*-F 5'-TAG AGT GTT GGT GGT GCT CTC TGT-3' (forward) and *PhGH*2-R 5'-CGA TAA GCA CGC CGA TGC CCA TTT-3 (reverse). The β-actin gene was used as a reference gene, and the primers were bact-F 5'-TAT GAA GGT TAT GCT CTG CCC-3' (forward) and bact-R 5'-CAT ACC CAG GAA AGA TGG CTG-3' (reverse). The sizes of the amplification products were 392 bp for the *PhGH* gene and 300 bp for the β-actin gene. For each cDNA sample were always amplified independently on the same plate and in the same experimental run in triplicate. The melting curve analysis showed that all reactions were free of primer-dimers or other non-specific products (data not shown). In order to examine the *Ph*GH copy number, generation of the absolute quantitative standard curve was necessary. First, we prepared a series of standard samples containing 3×10^1, 3×10, 3×10^3, 3×10^4 copies of plasmid pCcBA-*PhGH*. Copies number value ware calculated by the Rotor-gene Q software (Qiagen).

Determination of Enzyme Immunoassay for the Quantitative of Insuline-Like Growth Factor (IGF-I) in Plasma African Catfish Transgenic F-1

Plasma samples transgenic (n = 10) and non-transgenic (n = 10) were analyzed for Insuline-like Growth Factor (IGF-I). All samples were run in duplicate. Plasma samples were analyzed using IGF-I ELISA kit (DRG diagnostic, USA) and standard according to the protocol of the manufacturer.

RESULTS AND DISCUSSION

Detection and Distribution *Ph*GH in All Tissue

Distribution of *Ph*GH in all organs were shown in Figure 1. Transgene could be distributed and expressed in pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, caudal fin, gill, and eye, indicating that in transgenic fish happened over expression. Expression of *Ph*GH have been controled by β-actin promoter. β-actin promoter could active in all tissue and it didn't need out induction. No *Ph*GH signal was observed with cDNA from tissue of non-transgenic (controls). Mori & Devlin (1998) reported that the transgene was detected, distributed, and expressed in pituitary, liver, kidney, skin, intestine, stomach, muscle, spleen, pyloric caeca in fish coho salmon; in liver, gills, hearth, brain, skeletal muscle, kidney, spleen, intestine, and testes in tilapia (Caelers *et al.*, 2005; kobayashi *et*

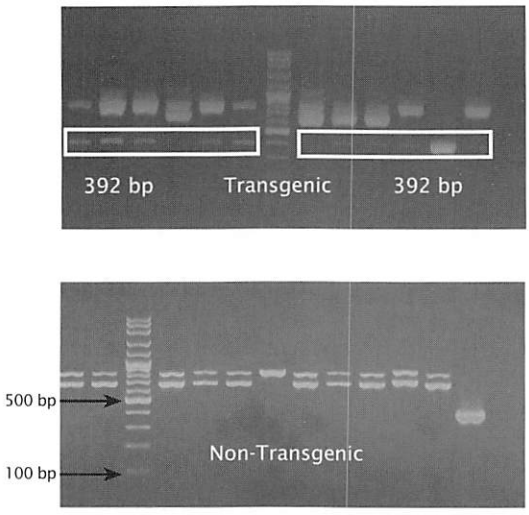

Figure 1. Detection of *PhGH* in several tissues from transgenic African catfish founder (F_0); brain (1), pituitary (2), muscle (3), intestine (4), liver (5), slpeen (6), kidney (7), thymus (8), gill (9), stomach (10), hearth (11), negative control positive control (-); M indicates a DNA marker (100-3,000 bp; Vivantis); the expected size of *Ph*GH gene the amplified fragment is 392 bp

al., 2007); in eye and brain in salmon (Devlin *et al.*, 2012). In addition, Farlora *et al.* (2009) reported that transgene (β-actin gene/EGFP) was distributed and expressed in the gills and operculum, caudal fin, muscle, gills, heart, liver, testes, and ovaries. Later, Caelers *et al.* (2005) have demonstrated that, transgene was expressed in brain, pituitary, gills, heart, gut, liver, muscle, kidney, spleen, and testes.

The *Ph*GH mRNA levels in the organs investigated were quite different, liver exhibited the highest level, in liver 23 x 10^6 copies was present in gills 0.5 x 10^6 copies while heart exhibited as little as 0.13 x 10^6 copies (Figure 2). High expression *Ph*GH mRNA in liver will induce the synthesis of insulin-like growth factor-I (IGF-I) and therefore it stimulates the muscles to synthesize protein. In addition to affecting the secretion of IGF-I, GH hormone can also directly stimulates the muscles to synthesize protein. This factor is thought to cause some transgenic individuals to have weight body than non-transgenic (Eppler *et al.*, 2007).

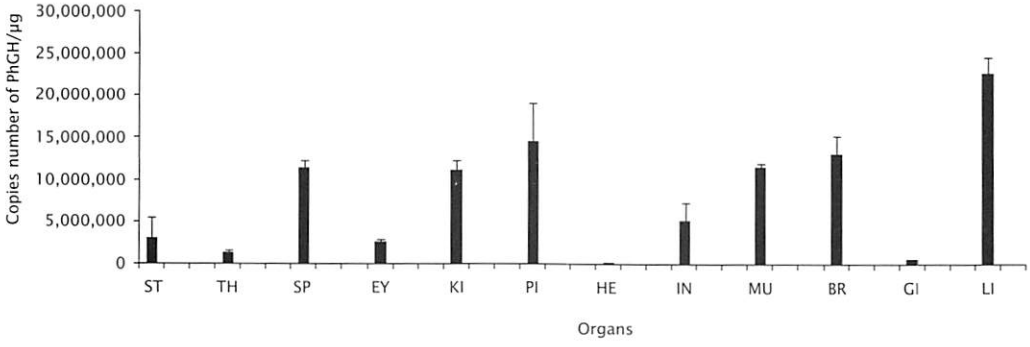

Figure 2. Analysis of quantitative RT-PCR for all tissue; vertical bars represent standard deviation for three fish; stomach (ST), thymus (T), spleen (SP), eye (EY), kidney (KI), pituitary (PI), heart (HE), intestine (IN), muscle (MU), brain (BR), gill (GI), and liver (LI)

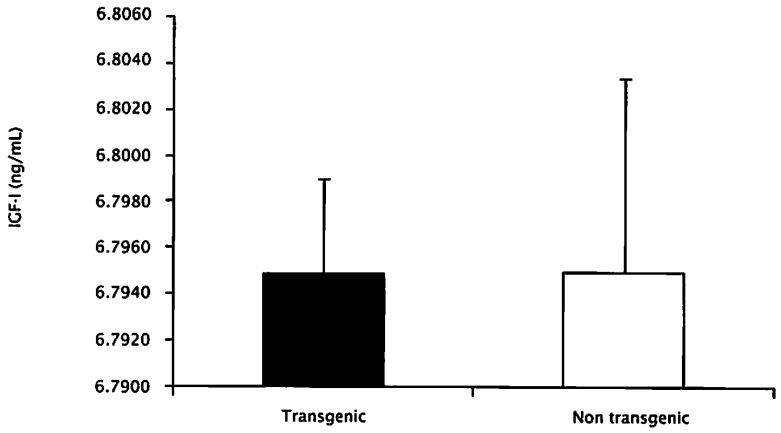

Figure 3. Analysis of quantitative of IGF-I in plasma. Vertical bars represent standard deviation for ten fishes

Determination of Enzyme Immunoassay for the Quantitative of Insuline-Like Growth Factor (IGF-I) in Plasma African Catfish Transgenic F-1

Using enzyme linked immunosorbent assay, the plasma IGF-I levels in transgenic individuals (6.7948 ng/mL ± 0.04 ng/mL) were not significantly different to non-transgenic (6.7950 ng/mL ± 0.08 ng/mL), despite increased *Ph*GH transgene expression levels in liver (Figure 3). The lower IGF-I serum levels or IGF-I levels comparable in the transgenic animals caused by the increased content of IGF-binding proteins (IGFBPs) in the liver of the transgenics may lead to retention of IGF-I by these BPs (Pierce *et al.*, 2001). The higher level of exogenous GH gene expression may not represent the production of a biologically active hormone (Figueiredo *et al.*, 2007).

CONCLUSION

*Ph*GH gene was detected and distributed in all organs of transgenic African catfish F-1. Highest level of *Ph*GH gene expression was found in the liver. The plasma IGF-I levels in transgenic were not significant with in non-transgenic individuals.

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