

**DISTRIBUTION AND EXPRESSION OF STRIPED CATFISH
(*Pangasionodon hypophtalmus*) GROWTH HORMONE GENE (*PhGH*)
IN THE ORGAN OF AFRICAN CATFISH (*Clarias gariepinus*)
TRANSGENIC FOUNDER**

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ABSTRACT

Faster growing African catfish can be produced by transgenesis. This study was conducted to investigate the distribution and expression of growth hormone gene (*PhGH*) in various organs of the transgenic African catfish (*Clarias gariepinus*) founder (F_0). Transgene was detected using the PCR method in various organs, namely pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, caudal fin, gill and eye. Transgene expression levels were analyzed using the method of reverse transcriptase-polymerase chain reaction (RT-PCR), β -actin gene used as internal controls. The results showed that the *PhGH* was detected and expressed in all organs of the transgenic African catfish founder. The high level of *PhGH* expression was found in the liver, pituitary, intestine and brain; smaller amounts were detectable in muscle, spleen, kidneys, heart, and stomach, caudal fin, gill and eyes, range from 0.02-0.75 *PhGH*/ β -actin mRNA. The expression levels of *PhGH* had positive correlation with tissue and body size ($P < 0.05$).

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

INTRODUCTION

Catfish is one of the potential aquaculture commodity in Indonesia, specifically African catfish, *Clarias gariepinus*. The total production of this species reached 242,811 tons in 2010 (FAO, 2012). Developing the catfish farm is expected to enhance the catfish industry and increases the national fishery product in the future. Improved genetic quality of both fish broodstock and seeds is considered as important components to support the achievement of the target. Genetic improvement through transgenic is one method that can be used to increase aquaculture productivity.

Transgenesis involves genomic alteration of an organism through insertion, modification or deletion of a gene with the objective of modifying characteristics of interest (Carter, 2004). In this manner, new stable and genetically determined characteristics can be incorporated into the genome of the receptor organism and possibly transmitted to the next generations. In the last two decades, this technology was successfully applied in fish due to the fact that these inferior vertebrates present reproductive and biological characteristics that allow easy manipulation of their genetic and physiological processes in the early stages

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of ontogenesis (Zhu & Shu, 2000). However, genetically modified fish have also been developed as experimental models for fish breeding research, specially in studies increasing aquaculture productivity using growth hormone gene transfer. Several transgenic fish have been successfully produced (Zhang *et al.*, 1990; Du *et al.*, 1992; Rahman & Maclean, 1992; Devlin *et al.*, 1995; Rahman *et al.*, 1998; Nam *et al.*, 2001; Kobayashi *et al.*, 2007). Growth hormone transgenes of fish origin have universally been shown to be more effective in growth enhancement than those of non-fish origin (Devlin *et al.*, 1995). Growth hormone, produced by the hypothalamus-pituitary-adrenal (HPA) axis, is a pleiotropic hormone regulating many aspects of fish physiology, including growth (Donaldson *et al.*, 1979), osmoregulation (Sakamoto *et al.*, 1997), and reproduction (Trudeau, 1997).

The transgenic technology producing faster growing catfish has been started since 2008 in Indonesia by microinjection (Gusrina *et al.*, 2009). The microinjection was used to produce transgenic fish. It almost invariably produces mosaic fish due to delayed transgene integration, which occurred only after a few cycles of embryonic cell division (Figueiredo *et al.*, 2007). However microinjection method used on catfish embryo has a weakness among others, required a high level of skill in the application. The injected eggs were often broken and attached to the needle of microinjection, and the number of embryos that successfully hatched and lived to become adults were very low compared to number of eggs produced fish so the chances of getting the broodstock are able to transmit the transgene at very low on the next generation (Stuart & Westerfield, 1988).

Nowadays, an alternative way of expressing transgenes in fish has been developed using electroporation to introduce transgenes into the male germ cells. This method has been developed to increase the efficiency of transgene integration or to produce a large number of transformed individuals simultaneously. The electroporation method has been successfully performed to express the transgene in sperm cells and is capable of producing a number of individuals in the same time (Powers *et al.*, 1992).

Early studies were conducted with GH gene transfer in the African catfish. Gene con-

structs used were "all fish" GH gene of striped catfish (*PhGH*) and β -actin promoter from carp (pCcBA) using the method of sperm electroforation which acted as carriers of the transgene. Transgenic fish were produced by fertilizing wild-type eggs with sperms collected from transgenic African catfish containing the pCcBA-*PhGH*. The results showed that growth effects can be observed, 2-fold relative to controls (Dewi *et al.*, 2011). The growth effect of transgenic African catfish founder has been due to the distribution and over expression of *PhGH* gene. The aim of this research is to evaluate distribution and expression *PhHG* growth hormone gene in transgenic African catfish organs.

MATERIALS AND METHOD

Animal and Sample Collection

Three of transgenic fish (founder) and three of non transgenic fish were analyzed in order to detect of *PhGH* in all tissue. The transgenic founders (F_0) African catfish (8 month of age) carrying the pCcBA-*PhGH* construct were generated by electroporation with 100 μ g/ml of the construct. 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* All Organs

DNA Extraction

Genomic DNA of each individual tissue 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 μ L of lysis digestion buffer, 20 μ L of proteinase K, followed by incubation in 56°C for 2 hours and adding 20 μ L of RNase A solution followed by incubation in room temperature for 10 min. The lysate was added 200 μ L with lysis solution followed by vortexing for 15 second. The lysate DNA was then precipitated by adding 400 μ L 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 μ L of washing buffer 1 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 μ L 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 μ g/mL) and viewed using gel documentation system ultraviolet transillumination.

Polymerase Chain Reaction (PCR)

Amplification of *PhGH* was performed using thermal cycling system (mycycler, Biorad). PCR standard was performed in a final volume of 25 μ L, using a commercial kit master mix faststart PCR master (Roche, Germany). The PCR was used to amplify a 390-bp fragment with primers *PhGH1-F* (5'- TAG AGT GTT GGT GGT GCT CTC TGT -3') and *PhGH2-R* (5'- CGA TAA GCA CGC CGA TGC CCA TTT -3'), for 35 cycles: denaturing at 94°C for 30 secs, annealing at 53°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 48°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 μ g/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, 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 (1mL per 50-100 mg tissue) followed by incu-

bation for 5 mins at room temperature. After added 100 μ L 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 μ L RNasefree (Ambion) and heated at 60°C for 10 mins. Total RNA was diluted to 1.0 mg/mL in RNase-free water. For synthesizing 1st-strand cDNA, 50 μ g of total RNA was treated with commercial 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 μ L.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using thermal cycling system (mycycler, Biorad). The RT-PCR was formed to detect exogenous DNA *PhGH* in pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, gill and eye. Two microliter cDNA was used as a template for RT-PCR and then mixed with 1 μ L primers *PhGH-F* (5'- TAG AGT GTT GGT GGT GCT CTC TGT -3') and *PhGH2-R* (5'- CGA TAA GCA CGC CGA TGC CCA TTT -3'), for 35 cycles: denaturing at 94°C for 30 secs, annealing at 53°C for 30 secs, and extension at 72°C for 1 min which produce a 390 bp *PhGH* fragment but does not amplify the endogenous African catfish growth hormone gene and using cDNA β -actin as an internal control. PCR products were separated on 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 μ g/mL) and visualized by ultraviolet transillumination.

Semiquantitative Analysis of RT-PCR Products

Semiquantitative analysis was done according to the method described by Laing *et al.* (Laing *et al.*, 2001). In order to adopt a more semiquantitative approach to analyze exogenous *PhGH* gene expressions, both exogenous *PhGH* genes and β -actin genes were amplified using a series of cycle numbers (21-30). Following this procedure, for each gene under analysis, the optimal cycle number (do not reach plateau) was determined and subsequently employed in the above expression analysis. After determining the optimal cycle

number, specific PCR was conducted three times. *PhGH* genes (25 cycles)/ β -actin (25 cycles) ratio was determined by using Total Lab™ 1D 21 CFR v12 software (Total Lab Ltd, United Kingdom).

Statistical Analysis

Values for mRNA are expressed as means \pm SD. The statistical significance was determined by correlation expression *PhGH* with organs and bodies size SPSS vers. 16. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Detection and Distribution *PhGH* in All Tissue

Detection of *PhGH* in all organs was shown in Figure 1.

All transgenic fish were found to have transgene *PhGH* in all tissue (392 bp). However non transgenic only detected endogenous gene (600 bp). Exogenous (GH) gene was detected and distributed in pituitary, liver, kidney, skin, intestine, stomach, muscle, spleen, *pyloric caeca* in fish coho salmon (Mori & Devlin, 1998), liver, gills, hearth, brain, skeletal muscle, kidney, spleen, intestine and testes in Tilapia (Caelers *et al.*, 2005), eye and brain in salmon (Devlin *et al.*, 2012).

Analysis Expression of *PhGH* in Various Organs

Analysis of transgenic fish by *reverse transcriptase*-Polymerase Chain Reaction (RT-PCR) revealed that transgene could be detected in pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, caudal fin, gill and eye (Figure 2). As expected, no band of transgene origin was amplified from mRNA non transgenic organs, indicating that no transgene *PhGH* expressed in non transgenic organs.

We found that all transgenic founder detected and expressed *PhGH* gene to all examined tissue, indicating that *PhGH* were integrated into sperm cell. The integration exogenous in the sperm genome due to the internalized exogenous sequences reach the nuclear scaffold of sperm cells; therein, they were subjected to rearrangement(s) mediated by endogenous nucleases and undergo recombination events that eventually caused their integration in the sperm genome

(Spadafora, 2008). Integration in the host genome is rarely under these conditions, and so far it has only been claimed by medaka transgenic fish (Thermes *et al.*, 2002).

PhGH expression level in transgenic founder of African catfish organ was analyzed by semi quantitatif PCR. Expression *PhGH* in liver is higher than another organs (Figure 3). Organ in transgenic catfish are heavier than non transgenic, indicate that over expression *PhGH* in transgenic African catfish organs ($P < 0.05$) (Figure 4). High concentrations of GH hormone in the fish body will induce the synthesis of IGF-I in the liver 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 larger body and organ size than non-transgenic (Caelers *et al.*, 2005).

No mosaicism *PhGH* gene was detected in organs from transgenic African catfish founder, *PhGH* gene was expressed in all organs with different levels. In theory, if all the germ cells of a transgenic fish contained transgene (*i.e.* no germ cell mosaicism) and this fish was mated with a wild-type fish, 50% of the offspring would expression the transgene (Stuart & Westerfield, 1988).

The GH expression from all tissue was classified according to their GH expression patterns Gibbs and Schmale (Gibbs & Schmale, 2000; Thermes *et al.*, 2002) as follows: weak = few cells expressing GH; moderate = less than 50% of the body expressing GH; or strong = more than 50% of the body expressing GH. Further studies examining the developmental profiles of expression are required to determine whether a correlation exists between transgene expression and growth rate.

CONCLUSION

PhGH gene was detected and distributed in all organs of transgenic African catfish founder. Highest level of *PhGH* gene expression was found in the liver. The expression of *PhGH* showed correlation between levels of expression *PhGH* and size of organ and body.

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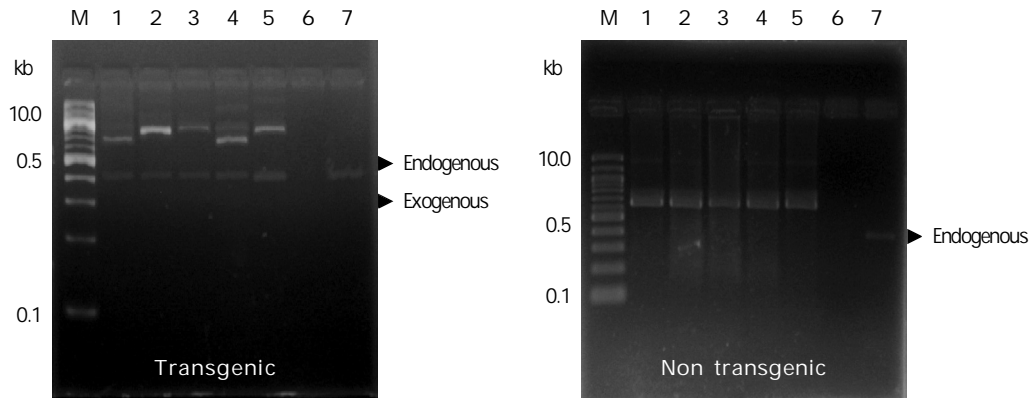


Figure 1. Detection *PhGH* in several tissues from transgenic African catfish founder (F_0). Brain (1), pituitary (2), muscle (3), intestine (4), liver (5), negative control (6), positive control (7). M indicates a DNA marker (0.1–10.0 kb, Vivantis). The expected size of exogenous *PhGH* the amplified fragment is 392 bp

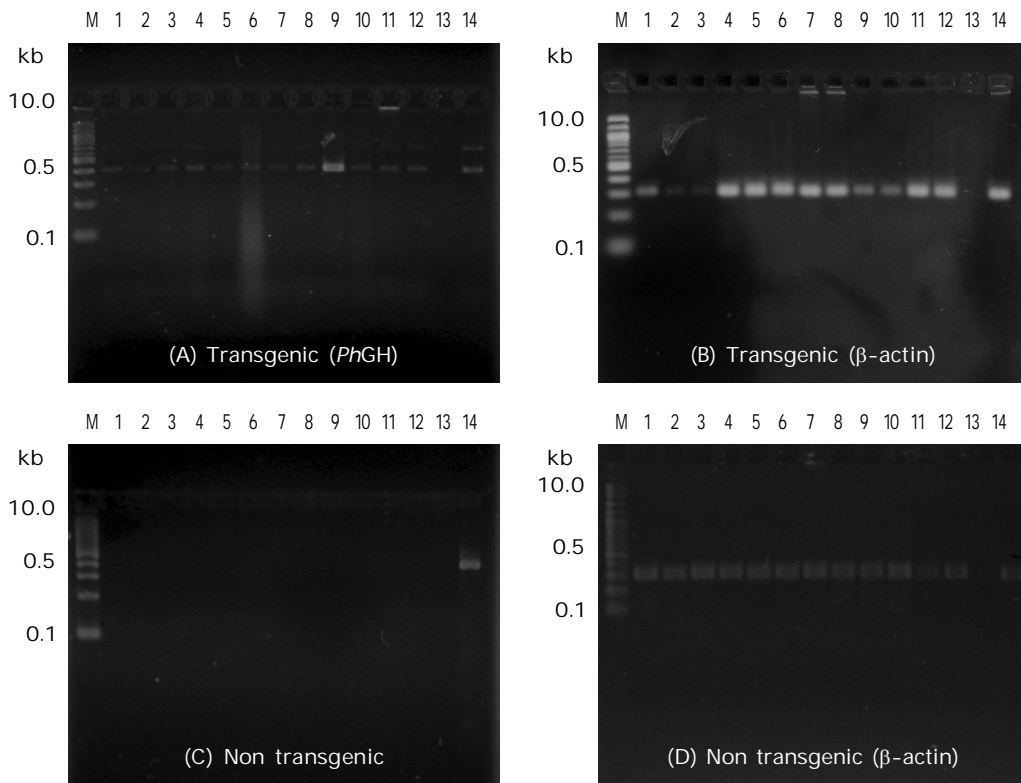


Figure 2. RT-PCR analysis of total RNA in all organs from transgenic African catfish founder (F_0). Transgenic (*PhGH*) (A), transgenic (β -actin) (B), non transgenic (C), non transgenic (β -actin), brain (1), pituitary (2), muscle (3), stomach (4), eye (5), gill (6), heart (7), intestine (8), liver (9), spleen (10), kidney (11), caudal fin (12), negative control (13), positive control (14). M indicates a DNA marker (0.1–10.0 kb, Vivantis). The expected size of the amplified fragment was 392 bp. β -actin was used as an internal control

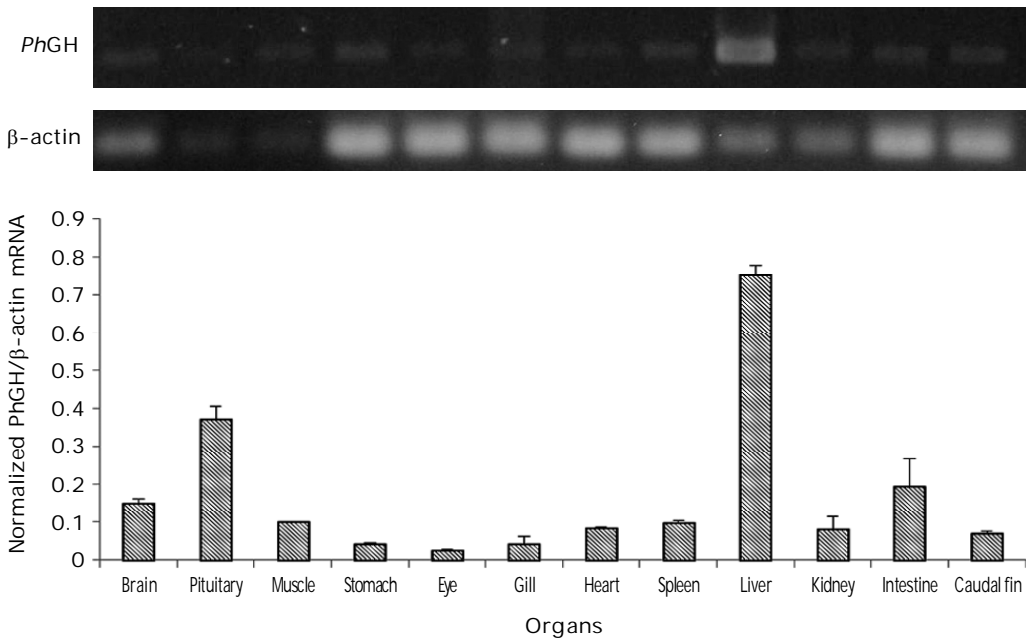


Figure 3. A semiquantitative analysis of RT-PCR in all tissue. Vertical bars represent standard deviation for three fishes

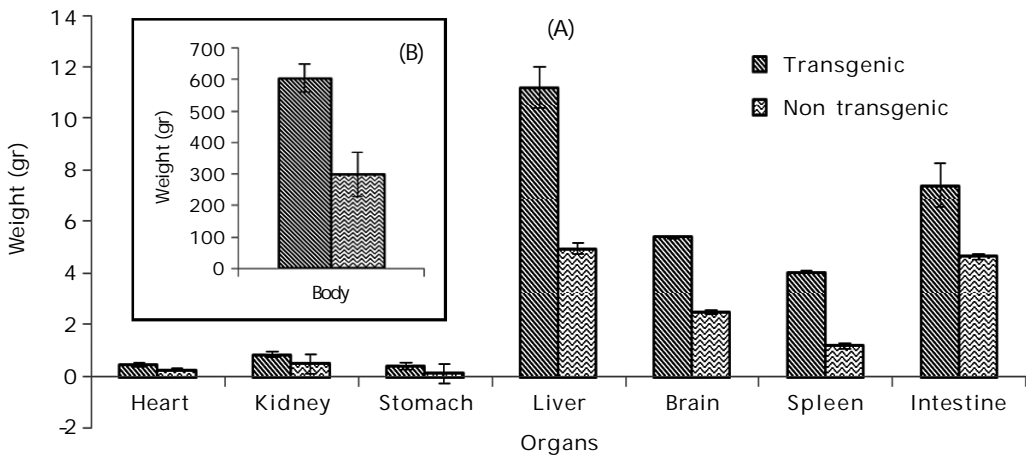


Figure 4. (A) Several organs and (B) body size between transgenic and non transgenic (control African catfish). Vertical bars represent standard deviation for three fishes

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