DETECTION, TRANSMISSION, AND EXPRESSION cDNA GROWTH HORMONE GENE (*Ph*GH) OF STRIPPED CATFISH IN F-1 TRANSGENIC AFRICAN CATFISH

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ABSTRACT

In previous study, the fast growth transgenic founder of African catfish was produced harboring a growth hormone (GH) gene construct containing a stripped catfish growth hormone (*Ph*GH) cDNA. This study was conducted to investigate transgene (*Ph*GH) transmission and expression in F-1 transgenic African catfish. The transgenic founders (female) were crossed with non-transgenic (male) to produce heterozygous F-1 progeny. *Ph*GH gene was detected in the embryo, larvae, and seed of the transgenic F-1 using PCR method. Expression levels of transgene in embryo and larvae were analyzed using real-time quantitative PCR (qPCR) method. The transgene was detected in embryo, larvae and seed of F-1 transgenic African catfish. Founder could transmit *Ph*GH gene to transgenic F-1 lines in ranged 36% to 48%. Expression level of *Ph*gh gene in embryo was higher than that of the larvae; whereas in the embryo was 1.5 x 10^5 - 5.2 x 10^5 copies or 0.49-9.82 fold, while in the larvae was 1.1 x 10^5 - 2.5 x 10^5 copies or 0.19-5.80 fold.

KEYWORDS: African catfish, transgenic, growth hormone, transmission, gene expression

INTRODUCTION

African catfish is one commodity that has been widely cultivated in Indonesia. Industrial development of African catfish is expected to increase and improve the national fishery products in the future. Improvement of the genetic quality of the seed and broodstock are regarded as an important component to support the achievement of national fish production. It can be done by molecular and biotechnological approaches, one of them by accelerating the growth of fish by the transgenesis method. The application of the transgenesis method in the world's fisheries can improve the character, such as growth performance, disease resistance, and adaptation to a particular environment (Sarmasik, 2003).

Transgenesis is done by the insertion of genes, gene modification or degradation with the aim of modifying the corresponding organism specific characteristics. These characteristics will be genetically stable since incorporated into the genome of a particular organism and may be passed on the next generation (Carter, 2004). Transgenesis has been successfully carried out to accelerate the growth of fish by introducing the gene encoding growth hormone (GH). GH gene transfer has been ap-

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plied to several species of fish and proven to increase growth (Nam *et al*., 2001; Kobayashi *et al*., 2007).

The research on fast-growing fish using transgenic methods continue to evolve. In addition to the development of transgene integration methods, several studies on genomic integration and germline transmission of the transgene were carried out for transgenic fish. The results showed that the frequency of genomic integration and germline transmission of the transgene were typically only a few percent of each generation (Stuart *et al*., 1990; Culp *et al*., 1991; Lin *et al*., 1999). Transgene can be integrated in the somatic cells and germ cells. The transgene which can be Integrated in the germ cells, will allow to be transmitted to the next generation. Besides the detectable transgene, the transgene should be expressed in the next generation as in embryos, larvae, and seeds (Farlora *et al*., 2009; Kobayashi *et al*., 2007; Devlin *et al*., 2012).

Previous research has been conducted on the introduction of stripped catfish (*Pangasionodon hypophtalmus*) growth hormone cDNA (pCcBA-*Ph*GH) in African catfish (*Clarias gariepinus*), using the electroporation method. The founder was expected to inheritance the fast growth characteristics to the first generation (F-1). The transgenic founder have been crossed with non-transgenic to produce heterozygous F-1 progeny. This study aimed to determine the transmission and expression level of transgene.

MATERIALS AND METHOD

Animal and Sample Collection

In this study, the pCcBA-*Ph*GH construct (Dewi, 2010) and the transgenic (F_1) African catfish (four months of age) carrying the pCcBA-*Ph*GH construct which were generated by electroporation with 100 µg/mL of the construct (Dewi *et al*., 2011) were used. All of fish were from a single family of African catfish (*Clarias gariepinus*) and originated from the collection in the Research Institute for Fish Breeding, Indonesia.

Detection and Distribution *Ph*GH in Transgenic African Catfish F-1

DNA Extraction

*Ph*GH gene detection was performed on 25 embryos pooling, 25 larvae pooling, 255 fry each individual (2 months old), and organs (pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, thymus, gill, and eye). 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 consist of series of steps including cell lysis, DNA precipitation, binding DNA to the column, washing, and elution. To ensure the success of genomic DNA extraction process, the sample was run on mini horizontal gel electrophoresis. The sample was loaded into 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 commercial kit master mix fast start PCR master (Roche, Germany). The PCR was used to amplify a 392-bp fragment of *Ph*GH 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 seconds, and extension at 72°C for 1 min. The amplification using the specific primer of β-actin was performed as internal control. The β-actin primers: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 (Accession JF303887.1). 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 were then separated on 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 µg/mL) and visualized by ultraviolet transillumination.

Analysis of *Ph*GH Gene Expression Level

RNA Extraction

Total RNA was extracted from 25 embryos pooling, 25 larvae pooling, and organs (pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, thymus, gill, and eye) which were obtained 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). 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. 100 µL bromochloropropane per 1 mL Tri Reagent were added, then shaking for 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. The 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 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 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 performed to detect *Ph*GH gene expression in 25 pooled embryos, 25 pooled larvae and pituitary, brain, liver, heart, spleen, kidney, intestine, stomach, muscle, gill, thymus, and eye organs. The qPCR was conducted with 100 ng DNA 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 secs, followed by 40 two steps cycles at 95°C for 5 secs and at 60°C for 30 secs. The *PhGH*1-F and *PhGH*2-R primers were used. The β-actin gene was used as a reference gene. 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 always amplified independently on the same plate and in the same experimental was 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, a series of standard samples containing 3×10^1, 3×10^2, 3×10^3, 3×10^4 copies of plasmid pCcBA-*PhGH* were prepared. The copies number values were calculated by the Rotor-gene Q software (Qiagen).

Data Analysis

Data were analyzed descriptively and statistically. Descriptive analysis was conducted for detection and expression of *Ph*GH gene, and presented as means ± SD. Statistical analysis using the Student's t-confidence interval of 95% was performed for expression of *Ph*GH gene.

RESULTS AND DISCUSSION

Total of 56 positive transgenic founders carried the transgene (*Ph*GH) in the caudal fin, only 9 individuals (10.71%) have been carried the transgene in the gonad. The founders that carrying transgene in gonads have been used to generate transgenic African catfish F-1 generation (Figure 1, Table 1). Recent studies have shown that the four founders which carried transgene in the caudal fin were produced by microinjection method, only one founder carried the transgene in sperm (Hinit & Moav, 1999). Later, Rahman & Maclean (1999) observed that 118 positive male tilapia fish carried the transgene on the caudal fin; only seven fish carried transgene in the sperm.

Detection of *Ph*GH Gene in Catfish F-1 Generation

*Ph*GH gene was detected in all embryos and larvae of six pairs of founders (Figure 2). This indicates that *Ph*GH gene could be transmitted from all founders to offspring (Table 2). The results of this study were similar to those reported by Lin *et al*. (1999) almost all founders could transmit the transgene to the next generation.

Founder could transmit *Ph*GH gene to F-1 transgenic lines in ranged 36% to 48% (Table 2), indicating that the individual containing the transgene in only a part of their germ cell. Liang *et al*. (2000) have demonstrated that, germline transmission of transgenes in F-1 transgenic lines in ranged 18.2% to 55.5%. Figueiredo *et al*. (2007) have also demonstrated transmission of transgenes from founder to F-1 generation in zebrafish in ranged 2.2% to 42%. These results indicate that, all founders could transmit the transgene to offspring (Table 2). In contrast Stuart *et al*. (1988), reported that 20

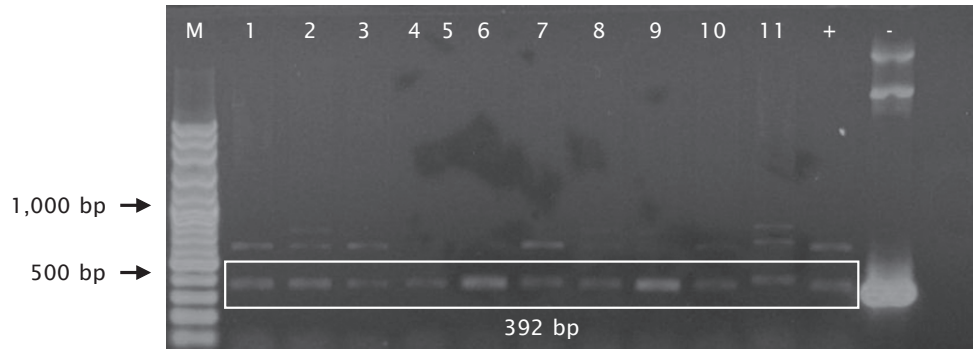

Figure 1. Detection of *Ph*GH gene in caudal fin and ovum from six of transgenic founder (F-0). Lane 1-5 = Caudal fin sample from founder and lane 6-11 = Eggs sample. M indicates a DNA marker (100-3,000 bp) (vivantis). (+) positif control (pCcBA-*Ph*GH). (-) negative control. The expected size of *Ph*GH gene was 392 bp

Table 1. Percentage of founders carrying *Ph*GH gene in caudal fin and gonad

Founder	Percentage of founders which have carried *Ph*GH gene in	
	Caudal fin	Gonads
Female	56.00% (56/100)	10.71% (6/56)

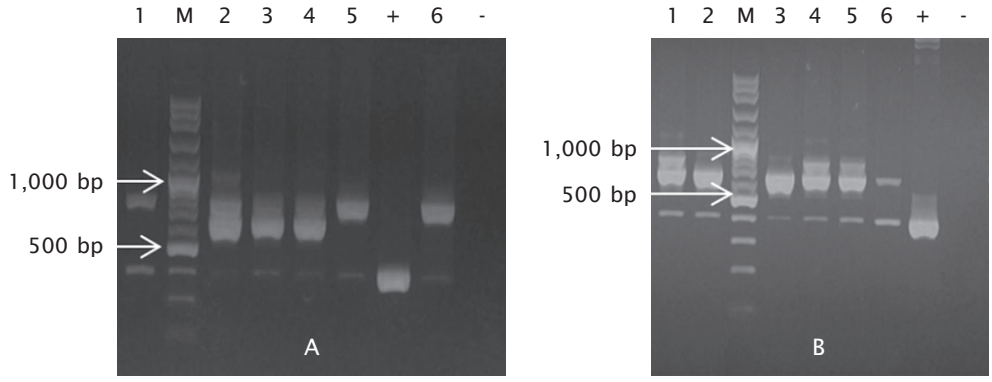

Figure 2. Detection *Ph*GH in embryo from catfish F-1 (A) and detection *Ph*GH in larvae from catfish F-1 (B). Lane (1-6) samples. M indicates a DNA marker (100-3,000 bp) (vivantis). (+) positif control (pCcBA-*Ph*GH). (-) negative control. The expected size of *Ph*GH gene amplification product is 392 bp

founder transgenic zebrafish were crossed with wild-type, only one founder could transmit transgene to the first generation. Rahman & Maclean (1999) have also reported that of the 13 founders tilapia were crossed to generate the F-1 generation, only three founders could transmit the transgene in the offspring with the percentage of transgene transmission in ranged 1% to 8%.

Analysis of Gene Expression in Embryos and Larvae *Ph*GH Catfish F-1 Generation Transgenic

Transgene could be expressed in all embryos and larvae of the six parents. Transgene expression level ranged from 1.5×10^5 to 5.2×10^5 copies in the embryos and 1.1×10^5 to 2.5×10^5 copies in the larvae (Figure 3 A and B).

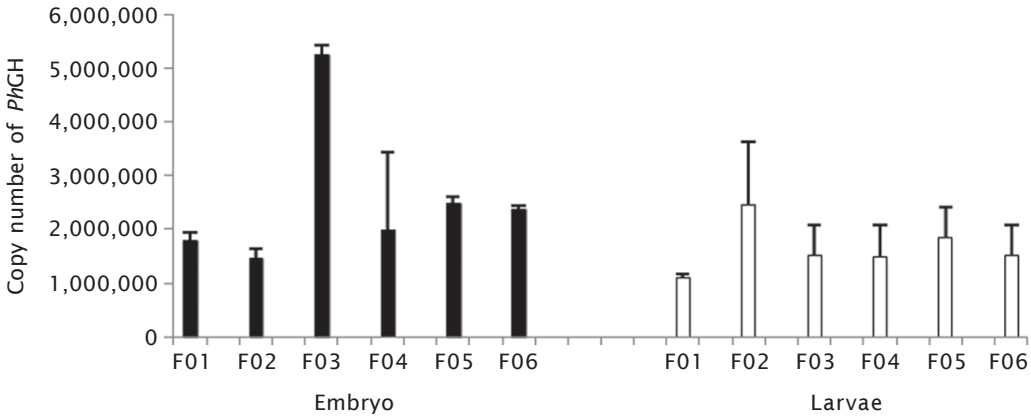

Figure 3. *Ph*GH gene expression level in embryo and larvae of F-1 transgenic African catfish analyzed by of quantitative RT-PCR. Vertical bars represent standard deviation (n = 3)

Table 2. Transmission of transgene in African catfish F-1 population

Transgenic founder	Percentage transmission transgene
F01	(50/22) 44
F02	(50/20) 40
F03	(50/24) 48
F04	(25/9) 36
F05	(25/2) 8
F06	(25/9) 36
Average	**38.22**

Transgene expression levels in each F-1 embryos and larvae varied. Level of transgene expression in embryos was higher than in larvae. The different expression was sometimes attributed to the use of promoter sequences from very distantly related species. Expression of GH transgenes has been documented (Devlin *et al*., 1995). Transgene expression in embryos and larvae also influenced by the carp β-actin promoter used in this study. Such research has been reported by Moav *et al*. (1992), β-actin promoter carp showed strong expression in common carp. β-Actin is an abundant protein in virtually all cell types and is very active promoter. Strong expression in zebrafish embryos has been identified by using β-actin promoter homolog (Higashijima *et al*., 1997). More recently, a study reported by Farlora *et al*. (2009), that the transgene could be expressed strongly in transgenic tilapia larvae at 10 days age after fertilazation using Japanese medaka β-actin promoter.

Furthermore, Inoue *et al*. (1990) reported that, the level of transgene expression varied among individual transgenic, due to variations of the copy number and different site integration in individuals. In addition, the average difference between the levels of expression caused by differences of number individuals carrying the transgene, it is necessary to test transgene expression per individual. Expression of *Ph*GH has been controlled by β-actin promoter. β-actin promoter can be active in all tissue and it does not need out induction. No *Ph*GH signal observed with cDNA from tissue of non-transgenic (controls).

CONCLUSION

The *PhGH* gene can be detected and transmited from the founder (F-0) to the first generation (F-1) with a transmission rate of 38.22%. *Ph*GH gene can also be detected and expressed in embryos and larvae. *Ph*GH level of gene expression in embryos was higher than in larvae.

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