Nig J. Biotech. Vol. 27 (2014) 34 – 39
ISSN: 0189 17131
Available online at http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org.

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Preliminary report on the production of transgenic *Oreochromis niloticus using tol 2 kit*

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(Received 09:11:12; Accepted 30:06:14)

Abstract

Pronuclear microinjection of donor plasmid containing the tol 2 transposon that was flanked by a promoter β- actin and green fluorescent protein (GFP) reporter gene were injected into the perinuclear cytoplasm of *Oreochromis niloticus* fertilized eggs. The aim was to determine if the β-actin promoter will drive the expression of the GFP in specific tissues of the embryo. Few days after hatching, GFP was strongly expressed in the embryos when observed under UV-light microscope. Subsequently, β actin fused with a foreign growth hormone gene in a donor tols 2 transposon along with messenger RNA was co-injected into early stage of fertilized eggs using micro injector. Out of the 30 eggs injected, 15 representing 50% hatchlings were obtained. GH was successfully transposed to the genome of Fo as confirmed from PCR of DNA from fin clip of the founder population. Growth performance in transgenic after 2 months of rearing was 1.06gm (3.7cm) compared with 0.68gm(3.5cm) in nontransgenic individuals. The founder generation is being raised to maturity to determine the level of efficiency in transmission to next generation.

Key words: Transposon, growth enhancement, *Oreochromis niloticus,* transgenesis, growth hormone gene

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Introduction

Transgenesis is the process of introducing an exogenous gene – called a transgene – into a living organism so that the organism will exhibit a new property and transmit that property to next generation. Several gene transfers have been successfully carried out in many fish species. e.g., Common carp, Rainbow trout, Atlantic salmon, *Clarias gariepinus*, Goldfish, Zebra-fish etc. (Liu, 1990; Moav, 1993, Rahman *et al*. 1998). The genes that have been transferred into fish include salmon or rainbow trout growth hormone, chicken 8crystalline protein, winter flounder antifreeze protein, etc. Transfection is achieved either by microinjection of the DNA construct into egg cytoplasm or by electroporation. Microinjection is performed within few hours after fertilization before nuclear division. Transfected ova are cultured in water where they hatch and raised.

Individual species like channel catfish, common carp, goldfish, rainbow trout, salmon etc that were injected with foreign growth hormone (GH) gene have expressed different degrees of growth increase. For example, carp showed up to 20% increase in body growth (Zhang *et al*., 1990); whereas the Nile Tilapia showed 33.3% increase (Rahman,1998). In addition to growth hormone gene transfer, many other genes have been successfully introduced and expressed in fish. Examples of these genes were antifreeze protein gene, a-globin gene, chicken o-crystalline protein gene, amongst others.

The possibility of producing bigger fish within a short culture period through transgenesis has been the major reason why this procedure is becoming popular. Apart from this, during embryogenesis, fish embryos exhibit external development and they have transparent body tissue making them to be highly adapted for transgenic procedures. There are many efficient methods of transgenic protocols, e.g. retrovirus infection and viral infection (Martinal *et al*., 2006), but they are not fully acceptable due to

concerns over safety issues. One approach that is free from biosafety issues involves the use of transposon systems.

Transposon elements are mobile DNA sequences that can be cleaved from the genome and inserted in another location through homologous recombination by a transposase enzyme that performs the "cut-and-paste" activity (Kawakami and Shima, 1999; Miskey *et al.,* 2005). Although transposons have been known for many years in plants and invertebrates, they have only recently been recognized and activated in vertebrates. According to Ivics (1997), the first transposon that was reconstructed from a Salmonid genome was called *Sleeping Beauty*. Kawakami and Shima (1999) have also isolated transposon from Medaka. Both transposons showed very weak host specificity and have proven to be active in many vertebrate classes, but they may be inactive in invertebrate. In several attempts, the use of transposons for transgenic fish production resulted in very high efficiency of integration of the target DNA into the host genome. This mechanism put forward my Kawakami (2004) is easy and a very effective method for the production of transgenic fish given high percentage of genome integration.

Today, zebrafish (*Danio rerio*) is the most widely studied fish model (Kimmel, 1989; Kimmel *et al*., 1995). The process of transgenesis through DNA microinjection into the cytoplasm of fertilized eggs is well established in the species. However, many commercially important species of fish present problems like opaque and tough chorion, unpredictable spawning time and long incubation and hatching times. These are reasons why in species like carp, tilapia and salmonid, transgenesis has only been accomplished in a few laboratories (Rahman and Maclean,1992; Rahman *et al*,1998). Even though tilapias are an important food species, transgenesis that will lead to significant enhancement in growth, disease resistant and other important traits in this species has not been perfected. Some attempts to inject linearized DNA constructs into tilapia eggs have been performed before first cleavage through the micropyle , but such a method often results in relatively low integration into the germline.

In transgenesis applications, different types of promoters have been used to drive the expression of the gene of interest and different levels of successes have been recorded. Examples of such promoters are β-actin, β-casie, prolactin, SV40 or RSV(*Rous sarcoma virus*) antifreeze protein promoter which are obtained from different species like carp and medaka (Liu *et al*.,1990; Moav,1993; Takagi *et al*., 1994). Chalfie *et al* (1994) used a modified *Xenopus* elongation factor 1α promoter to drive expression of the green fluorescence protein (GFP) reporter gene. In this study, attempt was made to first drive the expression of GFP with Zebra fish β-acting promoter. Then, the zebra fish β-acting promoter was used to drive the expression of tilapia GH in *O. niloticus* (homologous origin).

Materials and Methods

Isolation and Cloning of Zebra B Actin Promoter: Zebra β-actin promoter sequence was amplified from genomic DNA extracted from fin clips of Zebra fish following standard procedure (Rahman and Maclean, 1992). The amount of DNA was then quantified using ND-1000 Nanodrop Spectrophotometer following manufacturer's direction (Thermo Fisher Scientific). Two primers were designed from published sequence to amplify the β-actin promoter. PCR reaction was carried out in 50µl vol. containing 1µl of forward primer, 1µl of reverse primer, 1µl of Taq polymerase(Advantage), 3µl of DNA template, 5µl of buffer, 1µl of dNTP and 38µl of UPW. The reaction started with an initial denaturation at 94^{o}C for 2min followed by 30 cycles at 94^{o}C for 30s, 65^{o}C for 30s and 72^{o}C for 2mins; then a final elongation step of 72^{o} C for 3min. PCR products were separated by electrophoresis on 1% agarose gel containing ethidium bromide and photographed under ultraviolet light. The band of desired size was excised and purified using Promega Wizard® SV Gel and PCR Clean-Up System. The purified plasmid DNA fragment was sub cloned into pGMT vector. The plasmid was identified by PCR amplification and sequenced.

Construction of Zebra B Actin-Gfp Plasmid: PCR was carried out using primers to add att sites to the ends of DNA fragments following Invitrogen description. As a result of the sequence obtained from above, the attB4(GGGGACAACTTTGTATAGAAAAGTTGNN) and attB1R(GGGGACTGCTTTTTTGTACAAACTTGN) site adaptors were added to the promoter's primers (P1 & P2). A PCR reaction using the primers with the adaptors was performed using the Kapa HiFi polymerase. The reaction volume was 50µl containing 3µl of forward primer, 3µl of reverse primer, 1µl of DNA polymerase, 1µl of DNA template, 10µl of buffer, 1.5µl of dNTP and 30.5µl of UPW. The reaction started with an initial denaturation at 94^{o}C for 2min followed by 30 cycles at 94^{o}C for 30s, 65^{o}C for 30s and 72^{o}C

for 2min; then an extension step of 72°C for 3min. Immediately after the PCR reaction, the product is used for BP reaction of 10µl final volume made up of 0.7µl of PCR product, 1µl donor vector (#219), 6.3µl Tris buffer and 2µl BP clonase II enzyme mix (Invitrogen). The reaction was carried out at 26°C overnight. The reaction was stopped by adding proteinase K, and 2µl of the mixture was transformed and cloned using DH5α bacterial as described by Invitrogen. Positive clones shown as clear colonies were picked and amplified in a PCR using the Kapa HiFi polymerase (ORNAT) as described above. The PCR product was analyzed on agarose gel containing ethidium bromide and photographed. The Plasmid DNA was isolated and purified from positive colonies using a QIAgen Mini prep kit (Qiagen). DNA quantity was determined and sequenced.

This LR reaction was carried out to construct β–actin- GFP plasmid to test the activity of the promoter before final construction of plasmid with the growth hormone. The LR reaction of 10µl final volume made up of 0.25µl (β-actin in #219), 0.25µl(EGFP in #383), 0.4µl 3'poly A, 6.75µl Tris buffer, 0.4µl of #395 and 2µl LR clonase enzyme mix(Invitrogen). This construct was prepared for injection in a 10µl reaction of 1µl DNA (β-actin-EGFP),1µl transposase RNA, 1µl 1M KCl, 1µl Phenol red and 6µl UPW H_2O.

About 50pg of the plasmid was injected into each embryo. Embryos were kept in mini Zugler vessels until final yolk absorption. Eggs were kept constantly rolling due to upward current of water pumped from a water pump submerged in a water tank maintained at 27°C. Embryos were examined for GFP expression using inverted fluorescence microscopy and photograph was taken.

If expression proves sufficient, new vectors will be constructed by LR reaction to drive tilapia GH expression in the desired tissues.

Isolation and Cloning of Tilapia GH: The Nile tilapia growth hormone was amplified from pituitary cDNA of *O. niloticus*, using forward primer P3=CTCGCCCGCAAACAGAGCCT and reverse primerP4= CAATGCAACACATTTATTTCACAGATAACA designed from previously published sequence of tilapia GH. PCR was performed using the Red Load Taq Master (ORNAT). DNA fragment from the two products were sub cloned into pGEM®-T easy vector system and transformed using *E. coli* strain DH5α competent cell strain. This mixture was incubated on ice for 15 to 30 min, heat-shocked at 40 – 43°C for 50 sec, and returned to ice for 2min. One ml of Luria-Bertani (LB) broth, was added to the mixture and placed in a shaking incubator at 37 °C for 1.5 h. The cells were spinned for 5 min at 4000PPM and 900ul LB was discarded. The remaining 100ul was properly mixed and then cultured overnight on LB-amp medium at 37°C. Positive colonies were identified by PCR using Red Load Taq Master (ORNAT) and according to manufacturer's instructions; initial denaturing step of 94°C for 2mins, and followed by 35 cycles at 94°C for 30 sec, 65°C for 30 sec, 72°C for 1min, with final extension of 72°C for 2min. Plasmid DNA was isolated and purified from positive colonies using a QIAgen Mini prep kit (Qiagen). Sequence result of GH was analyzed.

PCR reaction using the specific primers with attB1 and attB2 adaptors was performed using the Kapa HiFi polymerase. The product was gel purified and cloned into the appropriate donor plasmids by a BP reaction. All Gateway methods and protocols were performed according to Invitrogen Multisite Gateway manual. The PCR products will be confirmed on gel and product will be sequenced.

The LR reaction for the construction of the final entry clones comprising the β actin – GH-poly A in pDest tol 2 was made in a 10µl final volume reaction made up of 0.24µl β-actin (in 299), 0.4µl GH (in 218), 0.4µl 3'poly A, 6.6µl Tris buffer, 0.4µl of pDONR #394 and 2µl LR clonase enzyme mix (Invitrogen). This construct was prepared for injection in a 10µl reaction of 1µl DNA (GH), 1µl transposase RNA, 1µl 1M KCl, 1µl Phenol red and 6µl UPW. Eggs were injected and incubated as described before. After two months, DNA was extracted from fin clip of tentative founder (n=5) and PCR carried out to confirm integration of GH sequence using appropriate primers (FP=Zf β-actin ACCCGTTGAAATGCGGTTGT and RP=CAATGCAACACATTTATTTCACAGATAACA).

Results and Discussion

Transgenic *O.niloticus* generated by micro injection of β-acting-GFP construct expressed strong ubiquitous fluorescence (Plate 1). Out of a total of 19 injected embryos, 15 representing 78.9% were positive. GFP was expressed all over the body of the embryo. This confirmation fully revealed that if we use the promoter to drive our growth hormone gene, it will successfully drive the expression of the

foreign gene. This result is consistent with observation of Higashijima *et al*., (1997), Amsterdam *et al*. (1995). Many transgenic fish species like *Cyprinus carpio* (Zhang *et al*, 1990); *Ictalurus punctatus* (Dunham *et al*., 1987); *Clarias gariepinus* (Volckaert *et al*.,1994) have been produced. However, this is the first known attempt to produce transgenic *O. niloticus* with foreign growth hormone gene using tol 2 kit. Positive results was obtained in the construct to generate transgenic *O.niloticus* using β-actin promoter which led excellently to good expression of the reporter gene GFP in the body tissue(Plate 1).The positive expression further confirms the long standing belief that closely related promoter sequences will usually lead to positive transgenic result (Rahman *et al*.; 1998). DNA has been extracted from *O. niloticus* fin clip and tGH was amplified in one the five founder using appropriate primers for amplification of tilapia growth hormone and β- actin promoter (P3 and P4).

At the age of 2 months, growth performance of the transgenic was 1.06gm (3.7cm) compared with the nontransgenic that was 0.68gm (3.5cm). The body depth in the transgenic is higher than what we have in the nontransgenic (Plate 4).The transgenic is being raised to maturity to cross with wild type fish to generate, on the average, about half population of transgenic line. Similar enhancement was reported in transgenic *O.niloticus* injected with growth hormone produced from salmon growth hormone gene driven by pout antifreeze promoter (Rahman *et al*., 1998) The expression of improved growth performance will eventually lead to reducing the culture period for farmers thereby leading to reduction in the maintenance and production cost. Transgenesis is a very useful method of improving tilapia growth especially if sterile populations are produced. This could be by crossing diploid transgenic individuals with tetraploid wild type to produce triploid. Apart from growth enhancement through the foreign growth gene, energy for growth is diverted into somatic development in sterile individuals. Transgene could also be produced to study gene expression and increase aquaculture production.

Conclusion

The founder population will be raised to sexual maturity and crossed with wild population to confirm if the transgene will be transferred to F1. Attempt would be made to raise the fish in a system that will allow the fish to grow to its full capacity and give better result on growth rate. Enhanced growth rate will transform into great improvement in fisheries of over 60 sixty countries where tilapia production is prominent.

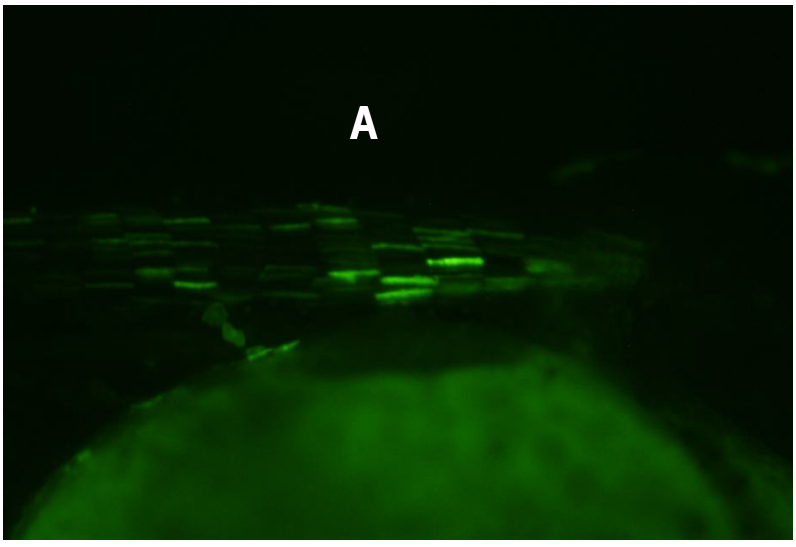

Plate 1. Fluorescent images of β-actin expression of GFP in transgenic embryo body tissues of O.niloticus*(mag. X100)*

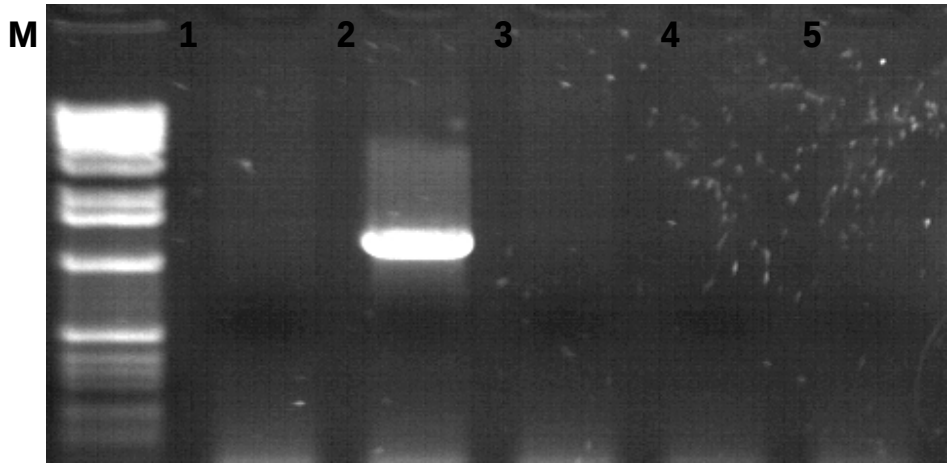

Plate 2. PCR confirmation of integration of GH using specific primers of O. niloticus (M=100bp marker, 1-5 screened putative transgenic fish) *(mag. X100)*

Plate3: Photomacrograph showing non transgenic (top) and transgenic (bottom) O.niloticus showing size difference*.*

References

Chalfie, M., Tu Y., Euskirchen, G, Ward, W.W. and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. *Science 263: 802-805*.

Ivics, Z., P.B. Hackett, R.H. Plasterk, and Z. Izsvak, (1997). Molecular reconstruction of Sleeping beauty, a Tc1-like transposon from fish, and its transposition in human cells*. Cell* . 91(4): p. *501-510*.

Kawakami, K. (2004). Transgenesis and gene trap methods of Zebra fish by using the Tol2 transposable element. *Method cell Biol. 77*:*201-222*.

Kawakami, K. and Shima, A. (1999). Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision o f a nonautonomous Tol2 element in zebrafish *Danio rerio*. *Gene 240(1): 239-244*

Kimmel, C. B., Ballard W. W. Kimmel S. R., Ullmann B., and Schillin T. F. (1995). Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics 203:253-310.*

Kwan, K.M., Fujimoto E., Grabher C., Mangum B.D., Hardy M.E., Campbell D.S., Parant J.M., Yost, J.H., Kanki P.J and Chien, C. (2007). The Tol2kit: A multisite Gateway-based construction kit for Tol2 transposon transgenesis constructs. *Developmental Dynamics 236(11): 3088-3099.*

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A. R. Hackett, P. B. (1990). Functional analysis of elements affecting expression of the β gene of carp. *Mol. Cell. Biol*.10: 3432-3440

Moav, B., Liu, Z., .Guise, K. S. Faras, A.J. and Hackett, P. B. (1993). Regulation of expression of transgenic in developing fish. *Transgenics 2: 153-161*.

Miskey, C., Izsvak, Z. ,Kawakami, K. and Ivics, Z. (2005). DNA transposons in vertebrate functional genomics*. Cellular and Molecular Life Sciences 62(6):629-641*.

Rahman, M.A., Mak, R., Ayad, H., Alam, S., and Maclean, N. (1998). Expression of a novel piscine growth hormone gene results in growth enhancement in transgenic tilapia (*Oreochromis niloticus*). *Transgenic Research 7:357-369.*

Shin-ichi, H., Hitoshi, O., Naoto, U., Yoshiki, H. and Goro, E. (1997). High-frequency generation of transgenic Zebra fish which reliably express GFP in whole muscles or the whole body by using promoters of Zebra fish origin. *Developmental Biology 192:289-299.*