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Status and prospects of gene editing and transgenic in fishes

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Abstract

Recent advances in high throughout DNA sequencing technologies have revolutionized for better understanding of structure-functional relationships of genes in identifying trait-associated transcriptomes and their regulated gene expressions. Subsequent breakthroughs in gene editing technologies such as zinc finger nucleases, transcriptional activator-like effect or nucleases (TALEN) and CRISPR (clustered regularly interspaced short palindromic repeats) determined chromosomal loci so as to understand gene functions *in vivo*. Such editing technologies are now being implemented in many laboratories due to an affordable cost and easiness of techniques. Targeted gene delivery and disruptions are now not only restricted to standard cell lines or stem cells, but also primary cell lines and non-model agriculturally important species. Progress and implications of gene integration and disruptions in food fishes like salmon, carps, etc. will be highlighted. The positive impacts on myostatin gene (negative growth hormone regulator) disruption mediated muscular growth have been documented. Transposon mediated gene integration technologies for value-additions to small indigenous aquarium fishes by expressing attractive fluorescent color genes could be the future of rainbow revolution. Issues linked with further-tuning with regards to improved efficacy and specificity, while reducing off-target effects of gene editing tools will be addressed. There are health and environmental concerns with genetically modified organisms (GMOs). CRISPR/Cas9 mediated editing generates indels and hence supposed to be free from transgene-nontoxic and non-allergen. Scientific progress regarding to generate genetically modified carps; those could well be cultivated in a confinement and at the same time economically profitable; will be highlighted. Emphasis should be given for transfer these technologies from the laboratory to land for the development of a consumer-friendly sustainable farming system.

Key words: Genetically modified organisms (GMOs), gene editing, CRISPR/Cas9, transgenic fishes.

Introduction

Approval (by USFDA) of AquAdvantage salmon, a genetically modified (a growth hormone regulating gene) food fish, has ignited GMO research trend in large-bodied food fishes. Transgenic fish is essentially required for increasing fish production within limited cultivable land to feed phenomenally increasing human population globally, in addition to its potential implications producing medicinal products from teleosts. Growth hormone gene constructs were preferably selected to generate transgenic fishes due to its obvious growth enhancing potential *vis-a-vis* conserved DNA sequences (Lee et al. 2015). Other traits like disease resistance (Dunham 2009); abiotic adaptations (Guan et al. 2011) and FCR (Feed conversion ratio) improvement (Krasnov et al. 1999) were also thought for transgenics. Biosafety, food-safety and ethical issues remained major concerns of fish transgenic researches. To overcome these concerns, several strategic research orientations were implemented globally, which will be highlighted in later sections.

The field of fish biology is now experiencing a transformative phase with the progress of advent in the genomics as well as genetic engineering. In teleosts, genomics along with next generation sequencing technology have been helpful to delineate genomic information. Further, Genome-Wide Association Studies (GWAS) along with identification of the Quantitative Trait Locus (QTL) have revealed a mass of candidate genes linked to various phenotypic traits. Many marker genes associated with phenotype traits could not be derived successful due to false-positive results. High-throughput sequencing platform

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for genome or transcriptome sequencing resulted in discovery of novel genes or transcripts associated with some traits. Physiological functions of many genes including novel genes, are still remained unexplored. Those gene functions need to be validated before to use them in genome-wide association studies. Transgenic technology has the potential to validate their *in vivo* functions. Mass-scale functional studies on novel genes by RNAi are documented in few studies with limited success due to time-consuming and labor intensive nature. Highly efficient such as genome editing techniques are now available to better understand gene functions. In aquaculture sector, gene editing technologies offer great promises to understand or modify gene functions. It is now possible to target single or multiple genes of polygenic quantitative traits. The current status and future prospects of gene manipulation researches in fish depending upon the needs are focused in this review.

Genetically modified organism (GMO) researches in fishes

The first transgenic food fish rainbow trout (*Onchorhycus mykiss*) and ornamental goldfish (*Carassius auratus*) were generated. Subsequently, several years of triumph in the generation of transgenic fishes, till now, only single transgenic fish (AquAdvantage salmon) as a food fish approved by FDA and available in the market. Conversely, model fishes like zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and goldfish (*C. auratus*) are being immensely utilized for the basic biology studies (Clifford 2014; Gong et al. 2003; Ye et al. 2011). The GMO/Transgenic fish research is primarily undertaken in only few cultured species including trout, carp, salmon, tilapia, etc keeping production enhancement is the major objective in aquaculture. Salmon and trout are cash crops and earn good foreign exchange, while the others are rich sources of protein in native states. Globally, about 50 labs are engaged with transgenics in teleosts; out of which most labs belong to developed countries including India. Collaborative efforts with private companies are also in pipeline, aiming towards commercialization. In transgenic research/GMO, a growth hormone (GH) gene has mainly been preferred to deliver so as to increase in growth rate.

Transgenic food fish and experimental fishes

The “AquAdvantage Salmon” first transgenic food fish introduced in the market after critical evaluation by US Food and Drug Administration (FDA), having improved growth rate compared to natural form (Clifford 2014). The genes being used in transgenic fish research are growth hormone (GH), metallothionein (Mt), antifreeze protein (AFP), crystalline and regulatory genes such as promoters (Gong et al. 2003). The promoter sequences such as heat shock protein (HSP), myosin light polypeptide chain 2 (*mylz2*), keratin and metallothionein (mt) successfully utilized for various purposes to drive gene expression as reported (Asaduzzaman et al. 2013). The inducible and tissue specific promoters (eg. HSP70) were used to combat stress conditions (Halloran et al. 2000). The detect heavy metal (cadmium mercury, zinc and copper) contamination; metallathionin promoter was characterized from several fishes (Mao et al. 2012; Ren et al. 2006). The model fishes such as zebrafishes and medaka has been utilized to solve biological problems or drug discovery as reported globally. For example, medaka used for monitoring reproductive events via estrogenic vitellogenin (vtg) gene promoters linked with GFP as reporter (Zeng et al. 2005b).

Ornamental transgenics

The ornamental fishes having huge demand and market, thus, several research organizations are trying to develop modified variety of ornamental fishes using genetic as well as transgenic approaches. Those ornamental transgenic fishes being developed using selected color genes or tissue specific promoter to express those color genes. The earlier evidence reported generation of stable lines of zebrafishes using tissue specific promoter such as krt8 and mylz2 linked with color genes such as RFP, GFP, BFP, YFP and CYP (Gong et al. 2003). This has resulted in “GloFish’ having six attractive fluorescent color variants and commercialized and names as cosmic blue, electric green, starfire red, sunburst orange moonrise pink and galactic purple (www.glofish.com). Due to various properties of those model fishes such as short life span, external fertilization, used in many laboratories around the globe. The mylz2 promoter derived from the zebrafishes utilized in other species for ornamental transgenesis such as medaka (*O. latipes*), skirt tetra (*Gymnocorymbus ternetzi*), and rohu, *Labeo rohita* (Mohanta et al. 2014; Pan et al. 2008; Zeng et al. 2005a).

National status on transgenic fish production

In India, research on transgenic fishes is not new, it has been reported since 1980. In fishes, transgenic research was started in MKU (Madurai Kamaraj University), National Matha College, Kollam and

CCMB (Centre for Cellular and Molecular Biology), Hyderabad using borrowed constructs from other countries. In 1991, first transgenic fish was generated in MKU with GH constructs. Further, Indian Council of Agricultural Research (ICAR) also taken initiative to engage and promote transgenic fish research programme for development of auto-trangenics fishes such rohu and catla to avoid biosafety issues. This has resulted in several experimental transgenic fishes such as rohu, catfish and zebrafish. Those research depicted, auto-transgenesis is less controversy and safer. Transgenic fish productions, development of food fishes and novel colour ornamental fishes have been reviewed (Rasal et al. 2016).

In ICAR-CIFA, the group of scientists initiated to work on transgenic fishes and reported several papers with respect to the basic researches, gene therapy and transgenics (Barman et al. 2010; Mohapatra et al. 2010). The functional β-*actin* gene promoter of rohu carp, capable driving ubiquitous expression, was identified (Barman et al. 2015). The isolated β-*actin* gene promoter/regulatory region are conserved among carps. This promoter can be used in any species of interests for foreign gene expressions (Hall et al. 2007; Her et al. 2004; Kinoshita et al. 2000; Rembold et al. 2006). Contrary to this, the isolated of the rohu (*L. rohita*) myosin light polypeptide chain 2 (*mylz2*) promoter (1.2 kb) could be used for expression in targeted skeletal muscle (Mohanta et al. 2014; Barman et al. 2015).

Issues or concerns related to transgenic fish

The major obstacle in GM fish production was mosaicism i.e., fail to achieve ubiquitous expression. The various researcher reported that, this occur mainly due to random integration of transgene, delayed integration at 1-cell stage, degradation of transgene by host enzymes and failure of inheritance of transgene (Moreau et al. 2014; Wu and Zhu 2003). In order to achieve successful transgenessis in fishes, stable integration of foreign gene/DNA in 1-2 cell stage of fertilized eggs is necessary, so that to inherit transgene in future generations. To monitor successful integration of transgene during initial period several reported genes being utilized such as GFP, RFP etc. To improve integration efficiency, transposable vectors are being utilized in various labs and resulted in successful GMO (Ivics et al. 2009). So far, Tol2, the transposable elements (mainly discovered in the genome of the Japanese medaka fish) demonstrated its uses in production of transgenics fishes such as zebrafish and medaka (Ivics et al. 2009; Nishidate et al. 2007). Earlier evidence depicted GH transgene in common carp affects growth improvement and not able to achieve consistent growth among its progeny (Lian et al. 2013). Other risks were reported associated with transgenic fishes were ecological, ethical concerns. However, there is no report of effects of transgeneic fishes on wild fishes, but it may pose serious threat as predicted by several research groups. The containment strategy could be helpful to combat ecological as well as other damages in ecosystem. Those include confined rearing of transgenic fishes in defined area or to make sterile fishes (Su et al. 2015).

The triploids fishes are sterile can be produced by inter-crossing among transgenic diploid with tetraploids as reported in common carp (Zhu et al. 1985). But, it is also not proper techniques as transgenic fishes may escape into environment and breed with wild population and resulted in ecological damage. Also triploids may pose serious problems in fishes such as phenotypic effects which hinder the performance of the GMO. Although, to make GMO is quite feasible in aquaculture species as reported by several labs across world, but model research facility including bio-safety equipments/facility are not available due to lack of funding and support. The food safety and public perception is the major obstacles encounter in the fish transgenic research. To overcome those concerns modern techniques evolved to understand and modify gene/genome for trait improvement such as NGS and Gene editing technology.

Gene editing techniques (ZFN, TALEN/CRISPR-Cas9): Pros and cons

Advancements of the next generation sequencing technologies together with bioinformatics analysis have heightened the knowledge at genomic level. Recently, genome editing technologies made a significant impact in the field of biology to investigate biological questions. Gene editing tools such as zinc finger nuclease (ZFN), transcriptional activator-like effector nucleases (TALENs) and CRISPR (clustered regularly interspaced short palindromic repeats)/ CRISPR-associated nuclease were utilized to undertake mutation at precise location in the genome. Those technologies have also been successfully implemented both in model and non-model species with greater success. Thus, it is now possible to undertake in vivo physiological functions in living organisms. The utility of gene editing technologies in

the fishes revolutionized the field of fish genetics and biotechnology (Carroll and Charo 2015). In case of livestock including fish, applications of genome editing has just gained momentum.

Though three technologies have revolutionized researches in the field of biology, but each one has evolved with some pros and cons. The ZFN technology was proven to be successful in generating knock-out/knock-in cell lines targeting multiple loci (Gaj et al. 2013; Miller et al. 2007). Target-specific domains are relatively longer in size and created problems in designing tools, in addition to its number of off-target effects. The TALEN scores certain advantages because of higher specificity and lower off-target effects as compared ZFN techniques (Sanjana et al. 2012). However, its sensitivity towards methylated target DNA remains a concern (Huang et al. 2014; Wright et al. 2014). Currently, the CRISPR/Cas9 cassetteis are being extensively used across eukaryotes due to cost effectiveness, easiness, and efficiency. The off-target effects of CRISPR/Cas9 technology remain controversial (Cao et al. 2016; Cong et al. 2013; Hwang et al. 2013).

Gene edited fishes

The CRISPR/Cas9 techniques has been effectively utilized in tilapia to carry out mutation in the genes such as *nanos2*, *nanos3*, *dmrt1*, and *foxl2* for understanding their function and found impacts of mutation such as masculinization and germ cell-deficient gonads (Li et al. 2014). In medaka, *Sox3* gene was edited using ZFNs and thus it was possible to functionally link this with sex determination (Takehana et al. 2014). Other evidences were also reported to incorporate targeted mutagenesis (Ansai et al. 2014; Ansai and Kinoshita 2014; Ansai et al. 2012). ZFN mediated heritable targeted *MSTN* gene disruption led to doubling of muscle mass (Doyon et al. 2008; Meng et al. 2008). TALEN-HR as well as Cas9 mediated gene editing has been successfully applied in zebrafish (Auer et al. 2014; Gonzales and Yeh 2014; Hruscha et al. 2013; Kimura et al. 2014). Recently, *MSTNb* gene disruption using TALEN resulted in dramatic improvement in muscle growth in zebrafish (Gao et al. 2016).

The MSTN gene as negative growth hormone regulator via its mutation or disruption depicted dramatic muscle growth in cattle, buffalo, sheep, pig, and dogs (Yamada 2012). Recently, MSTN gene was characterized and targeted in other fish species, such as yellow catfish (Dong et al. 2014), bay scallop (Guo et al. 2012) and rainbow trout (Phelps et al. 2013). MSTN gene consists of various isoforms across species (Gong et al. 2003).

Recently, CRISPR/Cas9 gene editing technology has been successfully to target immune related gene TLR22 of rohu carp through HR-mediated gene integration technique (Chakrapani et al. 2016). This was aimed at developing model rohu carp. To improve growth performance in common carp (*Cyprinus carpio*), gene editing techniques such as TALEN and CRISPR/Cas9 technology have been used to disrupt muscle specific genes such as *sp7, runx2, spp1* and *mstn* (Zhong et al. 2016). These findings demonstrated regarding gene editing in farmed carps.

Indian perspectives and concluding remarks

Gene editing tools have successfully been used to engineer targeted chromosomal loci in animals and fishes. In aquaculture sector, genetic engineering is emerging as a powerful method for breeding of fish and shellfish. While, US Food and Drug Administration (FDA) declared that all animals whose genes/genomes have been intentionally altered will be examined for safety and efficacy in a process (Nature doi:10.1038/nature.2017.21331). The production of auto-transgenic fish could be better option with assistance of Cartagena Protocols and bio-safety regulations. The GMOs linked risks must be properly assessed with scientific basis. However, ecological impacts should be studied clearly to avoid damages to ecosystem due to GMO. Currently, FDA updated their existing guidance for GMOs to include genome editing within its scope, and are issuing it in draft form for public comment. The various researchers group have begun using CRISPR-Cas9 system to create knock-out/in animals including fishes with view of future applications. Incorporating single or couple of bases mediated by CRISPR/Cas9 tool may not be associated with food safety concerns, since single base changes naturally takes place from generation to generation due to environmental effects. Due to high degree of specificity, simple mechanism and low cost genome editing has become an attractive technique. Ethical and public policy issues continue to be centre of international debate in relation to genetic modification of animals including fishes. Thus, with the exiting strategies based on genome editing will be useful to reduce the burden of future requirement of fish food, tolerance to changing climate and diseases.

India is blessed with natural resources of

indigenous small fishes such as *Parambassis ranga*, *Chanda nama*, *Laubuca laubuca* (Indian glass barb) (Fig. 1a). Their natural habitat is in North-Eastern regions of India. These are freshwater fishes, mainly considered as weed fishes, even though these fishes are consumed by village people. Their morphometric features, mainly translucent bodies, have great potential to become experimental fishes. Additionally, it is possible to add value by generating transgenic fishes expressing attractive fluorescent colors. *L. laubuca* is a hardy fish with feeding behavior of planktons, artemia and pelleted fishes, whereas *P. ranga* and *C. nama* prefer only live feeds. The knowledge on their breeding behaviors is insufficient. The pre-requisite for generation of value-added transgenic fish is optimizing their breeding protocol within laboratory glass aquaria. These two aspects, pertaining to basic biology and reproductive physiology, are current focused research in our laboratory. Value-added ornamental transgenic indigenous fish species have potential to bring rainbow revolution. Tol2 transposon system could effectively be implemented to raise ornamental transgenics, since these are not food fishes. Ecological risks are also minimal since those would be reared within confined glass aquaria. We have succeeded in identifying HSP90β gene promoter from *Channa striatus*, a walking freshwater fish and highly tolerant to hypoxia. In the absence of suitable breeding protocol for above indigenous fish species; transgenic zebrafishes (Fig. 1b), massively expressing GFP reporter gene driven by the HSP90β gene promoter, have successfully been generated (patent application filed and data yet to publish).

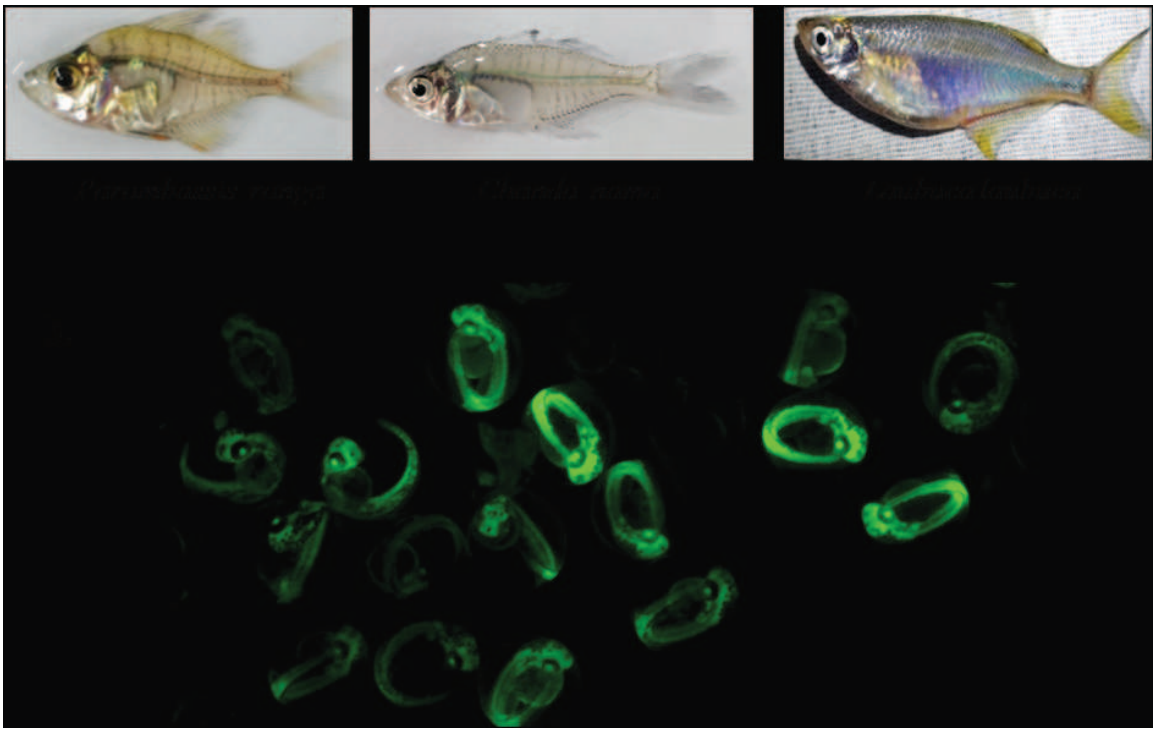

Fig. 1. Potential indigenous freshwater ornamental fishes:a). three fishes could be explored for generating value-added transgenics. b). Transgenic zebrafishes expressing GFP driven by fish HSP90β gene promoter

On the other hand, CRISPR/Cas9 technology could effectively be implemented to generate any gene of interests to generate model fishes. As stated in previous section, TLR22 gene was successfully knocked-out by HR mediated gene integration using CRISPR/Cas9 nuclease in large-bodied rohu carp. Therefore, it is possible to conduct physiological functions of a gene by raising model fishes. Other possible strategy could be either to disrupt myostatin gene and/or to replace myostatin gene by HR mediated integration of growth hormone gene in farmed carps. It is high time to demonstrate researchers to translate these technologies into farmers-friendly farming system.

Declaration

The authors declare no conflict of interest.

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