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REVIEW

Bigger, stronger, better: Fish transgenesis applications and methods

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Abstract

Transgenesis has been applied to several species to benefit from them in different fields. As natural fish stocks decline and the world population increase, the application of transgenesis on commercial fish species takes more attention to reduce the limitations of aquaculture and meet the increasing food demand. Transgenesis has been applied to obtain a stable transgenic line with improved traits to date. In aquaculture, growth rate, cold resistance, and disease resistance of commercial fish species were enhanced by transgenesis and even one of them, AquAdvantage Salmon, took place markets in the North America. Also, transgenic fish were developed to evaluate the health impacts of chemicals in ecotoxicology and provide more options with new color variants in ornamental fisheries. Different approaches for generating transgenic fish have been performed successfully, but they still require some developments. More transgenic fish could take place in the market by developing more efficient techniques and informing consumers about these techniques to reduce their concerns. This review discusses the application fields of transgenic fish with examples and provides an overview of gene delivery techniques and transgenesis methods.

Introduction

Transgenic animals are genetically modified organisms (GMOs) with heritable changes to the genome by integration of exogenous DNA (transgene) into the host genomic DNA. Since the human growth hormone gene has been successfully transferred to goldfish (*Carassius auratus*) genome to produce the first transgenic fish, more than 35 species had been genetically modified in research laboratories worldwide (Zbikowska, 2003; Zhu et al., 1985). Most transgenesis studies were conducted on fish traits for sustainable aquaculture by various gene delivery and transgenesis techniques (Durham et al., 1987; Ju et al., 2003; Sarmasik et al., 2002; Shears, 1991). Transgenic fish with enhanced traits could provide a great benefit as food for the growing world population while it could be a dangerous product of biotechnology upon escaping accidentally to the environment. However, aquaculture could more contribute to the food demand of the increasing world population by advanced biotechnology. There are many factors related to the environment (water supply and quality, environmental impact, climate change, farming technologies) and also with the cultured organism itself (feed supply, diseases) that can affect the development of aquaculture (Gómez, 2018).

Certain characteristics of the fish could be improved through transgenesis for more sustainable and profitable aquaculture. It is possible to save enormous money by increasing the growth rate of the fish as well as cold and disease tolerance. In addition to aquaculture, transgenic fish could be developed as experimental models for biomedical research (Goldman

et al., 2001; Ward & Lieschke, 2002). The use of transgenic fish in research laboratories as an alternative to rodents could considerably reduce the exploitation of mammals, decrease costs, and accelerate the research process (Zbikowska, 2003). Furthermore, transgenic fish could be used in environmental monitoring, and ornamental fisheries (Amanuma et al., 2000; Ju et al., 2003). In this review, we described application areas of transgenic fish in aquaculture and mentioned some gene delivery techniques. Furthermore, we attempt to provide an overview of both traditional and modern transgenesis methods.

Applications of Transgenic Fish

Transgenic fish in aquaculture

Growth enhancement

Insertion of genes for growth enhancement has been applied to many fish species using different DNA constructs relating to the origin of the growth hormone gene and the promoter. During initial experiments conducted on fish in the 1980s, DNA constructs comprised of mammalian or viral promoters and mammalian growth hormone genes, but then the genes and promoters of piscine origin were used (Durham et al., 1987; Nam et al., 2001; Rahman et al., 1998; Zhu et al., 1985). Although transgenes derived from very distantly related species had no or only modest effects on growth in transgenic fish, transgenes from piscine species were shown to be more efficient (Betancourt et al., 1993; Penman et al., 1991). Significant growth enhancement by genetically modifying fish with an exogenous growth hormone gene is achieved for a few species including nile tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), mud loach (*Misgurnus mizolepis*), coho salmon (*Oncorhynchus kisutch*), and rohu carp (*Labeo rohita*) (Barman et al., 2015; Devlin et al., 1994; Nam et al., 2001; Rahman et al., 1998; Zhang et al., 1990). Effect of transgene on growth rate could varied from 35-fold increase in size compared with the non-transgenic counterparts, to almost no difference depending on the species and DNA constructs (Nam et al., 2001; Pitkänen et al., 1999a).

Considerably high growth rate effects (typically 6- to 14-fold compared to controls) were observed for some salmonid species comprising Atlantic salmon (*Salmo salar*), Coho salmon, and Arctic charr (*Salvelinus alpinus*), but levels of growth enhancement were variable among transgenic salmonid lines (Devlin et al., 1994; Du et al., 1992; Pitkänen et al., 1999a). Extreme growth in salmonids carrying a transgene could be because of experiencing important seasonal reductions in growth while warm-water fish species naturally grow at near maximal rates during the year (Mori & Devlin, 1999).

Although constructs containing permissive viral or piscine constitutive promoters usually increase growth rate, some constructs could not affect growth performance. For example, a construct consisting of the growth hormone 2 gene from Atlantic salmon and the homologous promoter (SsGH2) did not affect the growth rate of rainbow trout (*Oncorhynchus mykiss*) expressing growth hormone only in the pituitary, the normal site of growth hormone expression (Pitkänen et al., 1999a). In a study, it was showed that growth enhancement could depend on the intrinsic growth rate and genetic background of the host strain (Devlin et al., 2001). Wild strains of rainbow trout naturally grow slowly while the growth rate is higher in selectively bred domesticated strains. Growth hormone construct was introduced into a wild and domesticated strain of rainbow trout, yet the introduction of the growth hormone construct into the domesticated strain did not cause further growth enhancement because this strain reaches maximal rate with the different genetic background (Devlin et al., 2001).

Upon DNA constructs were applied to fish, besides growth enhancement, other phenotypic changes in fish morphology, and some physiological abnormalities were observed in some species (Devlin, 1997). These could be an alteration of skin color, modifications of skull shape, acceleration of smoltification in salmonids, precocious sexual maturation, decreased fertility or even sterility, and reduced viability.

Several studies suggested that transgenic fish display considerable metabolic differences compared to non-transgenic siblings and their metabolism is more efficient (Krasnov et al., 1999; Martinez et al., 2000). For example, the juveniles of transgenic tilapia demonstrate increased protein synthesis and growth rate concomitant with enhanced glycolysis and oxidation of amino acids (Martinez et al., 2000). Also, transgenic charr showed enhanced metabolic activity and utilization of dietary lipids (Krasnov et al., 1999).

The story of transgenic Atlantic salmon:

The most well-known transgenic fish with an increased growth rate is the AquAdvantage Salmon developed by the group of Fletcher at Memorial University of Newfoundland (Gómez, 2018). This transgenic Atlantic salmon was created via microinjection of a DNA construct containing an antifreeze protein gene promoter from ocean pout (*Macrozoarces americanus*) and a Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone cDNA (opAFP-GHc2) into fertilized eggs of wild Atlantic salmon (Du et al., 1992). Therefore, the line of transgenic Atlantic Salmon has a copy of transgene expressing continuously along with its salmon growth hormone gene expressing seasonally. Transgenic Atlantic salmon reach market size (4-5kg) from eyed-egg stage in nearly 18 months compared to 36 months for conventionally farmed Atlantic salmon and also consume 25% less feed than conventionally farmed Atlantic salmon during the growth period (Gómez, 2018).

After a long journey through the US regulatory system, AquAdvantage Salmon is now produced by the company AquaBounty Technologies (Massachusetts,

USA). The journey started in 1993 when AquaBounty first approached the U.S. Food and Drug Administration (FDA) to find out the requirement for approval of AquAdvantage Salmon as food, but a regulatory pathway did not exist for genetically engineered (GE) animals (Van Eenennaam & Muir, 2011). The company appealed for regulation under FDA since they thought the difficult pathway for approval would contribute to addressing public concerns about food from GE animals. AquaBounty established an Investigational New Animal Drug (INAD) file with the Center for Veterinary Medicine (CVM) of the U.S. FDA to pursue the development of AquAdvantage Salmon in 1995. During the assessment period, AquaBounty Technologies constructed a land-based aquaculture facility (AquaBounty Panama) in the highlands of Panama to conduct trials of the Company's AquAdvantage Salmon (Aquabounty, 2020).

FDA released Guidance 187 for the regulation of GE animals in 2009 (Van Eenennaam & Muir, 2011). Based on this guidance, the FDA applies a hierarchical risk-based approach to evaluate GE animals and their edible products in seven-step by investigating the safety of the recombinant DNA construct for the animal, the safety of food from the animal, and any environmental impacts and efficiency claimed for the animal in the seven-step (Sanderson and Humphries, 2015). Molecular characterization of the rDNA construct should be conducted to detect whether it includes DNA sequences from viruses or other organisms that could cause health risks to the GE animal or those consuming the animal (Van Eenennaam & Muir, 2011). Also, molecular characterization of the GE animal lineage should be conducted to control the inherited stability of the rDNA construct in the next generations. Furthermore, the health state and development process of GE animals should be evaluated by phenotypic characterization comparing with non-GE animals (Sanderson & Humphries, 2015). If the GE animal is proposed as a source of food as with the AquAdvantage salmon, FDA evaluates the composition of edible tissues and risk of allergenicity compared to their non-GE counterparts (Van Eenennaam & Muir, 2011). FDA finally requires the preparation of an environmental assessment of the animal and of conditions suggested for raising the GE animal as stated in the product definition and the sponsor data supporting the claimed efficiency of GE animal (Van Eenennaam & Muir, 2011).

Following the release of the Guidance, AquaBounty Technologies submitted its final regulatory study to the FDA completing all tests for evaluation (Aquabounty, 2020). After that, AquaBounty's Panama site was investigated and approved by the FDA for the production of AquAdvantage Salmon. FDA concluded that AquAdvantage Salmon is safe to eat; and poses no threat to the environment under its raising conditions and then FDA convened its Veterinary Medicine Advisory Committee (VMAC) in a public meeting to review its findings (Van Eenennaam & Muir, 2011). The VMAC agreed with the FDA; AquAdvantage Salmon is safe to consume, and safe for the environment, therefore FDA completed a food safety assessment in 2010. The FDA consults with the National Marine Fisheries Service of NOAA and the U.S. Fish and Wildlife Service for more investigation on environmental impacts of AquAdvantage salmon in 2011(Aquabounty, 2020). These organizations agreed with the findings of the FDA that the AquAdvantage Salmon do not pose a threat to the environment. The FDA completed the environmental assessment and published in the Federal Register a preliminary Finding of No Significant Impact (FONSI) for AquAdvantage Salmon in 2012 (Gómez, 2018).

In 2011, AquaBounty Technologies also completed a New Substance Notification (Organisms) for AquAdvantage Salmon and applied it to Environment Canada. AquaBounty Technologies had continued its story in Canada by applying to the Canadian Food Inspection Agency in 2012 for registration of AquAdvantage Salmon as a Novel Feed and to Health Canada for a Novel Foods Pre-Market Submission (Aquabounty, 2020).

Environment Canada published a Significant New Activity Notice indicating AquAdvantage Salmon is not considered to be a risk to the environment in 2013 (Aquabounty, 2020). Therefore, AquaBounty Technologies receives authorization for the production of eggs at AquaBounty Canada's hatchery for commercial sale. However, Ecojustice on behalf of Ecology Action Centre and Living Oceans Society files lawsuit against the Canadian federal government (Health Canada, Environment Canada) and AquaBounty for permission to produce genetically modified salmon in Canada. Similar actions by non-governmental organizations also occurred when the FDA evaluating the environmental impacts of AquAdvantage salmon (Van Eenennaam & Muir, 2011).

The main environmental concern about AquAdvantage fish is the possibility of escape and cause the collapse of wild salmon populations. Environmental concerns could be mitigated by land-based production with physical confinement barriers and also biological measures resulting in 99% sterility and 100% female production stocks (Van Eenennaam & Muir, 2011). In principle, there is no difference between potential environmental risks related to the escape of transgenic fish and those related to the annual escape of a lot of farmed selectively bred fish. Matings between escaped farmed salmon and wild native fish could cause a substantial risk of extinction for natural populations, but the comparative risk of sterile transgenic AquAdvantage salmon is probably to be less than that of fertile, selectively bred, Atlantic salmon (Van Eenennaam & Muir, 2011).

AquAdvantage Salmon was approved by the FDA for consumption in the USA in 2015 (Gómez, 2018). After a while, the import of AquAdvantage Salmon into the USA was prohibited until labeling requirements were announced by FDA (Van Eenennaam, 2017). The

U.S. Congress passed a GMO food labeling bill and give 2 years to USDA to establish a labeling language and guidelines. Meanwhile, Health Canada approved the production, sale, and human consumption of AquAdvantage Salmon in Canada. Following those approvals, AquaBounty Technologies purchased certain assets of the Bell Fish Company LLC farm site in Albany, Indiana to establish the first commercial-scale production facility in the United States in 2017 (Figure 1) (Aquabounty, 2020).

The FDA approved to raise AquAdvantage Salmon at the Company's Indiana farm in 2018 (Aquabounty, 2020). After establishing of labeling language and guidelines for GMO food, the FDA allowed the Company to start farming AquAdvantage Salmon in Indiana in 2019. Also, the company gained permission from Environment and Climate Change Canada (ECCC) for the commercial production and grow-out of AquAdvantage Salmon in Rollo Bay facility. At the end of the tortuous journey, AquAdvantage Salmon reached to market in the USA as well as in Canada. The market of AquAdvantage Salmon would probably extend through projects conducted by AquaBounty Technologies in Brazil, Argentina, Israel, and China starting new journeys (The Fish Site, 2020).

AquAdvantage Salmon could also continue its journey in Europe. First, it should take proper scientific opinion from the European Food Safety Authority (EFSA) agency which provides independent scientific advice on current and emerging risks to food safety in the European Union (EU) (Slyck, 2017). A guidance on the environmental risk assessment of GE animals were published by EFSA to present information for applicants and risk assessors on placing GE animals on the EU market. EU regulatory system adopts precautionary principle for releasing of GE animals in constrast to FDA adopting prevention principle. Precautionary principle aims a high standard of environmental protection via preventative decision-making if risks are reasonably suspected. Based on this principal difference, AquAdvantage salmon is ban now in the EU, but this situation could change with the positive results of long-term effects of GE animals on enviroment and food safety, and also trade agreements (Debode et al., 2018).

Cold resistance

Many species of polar and northern fish inhabiting frigid water such as winter flounder (*Pleuronectes americanus*) and ocean pout produce antifreeze proteins to protect them from freezing (Lee et al., 2013). Based on their structural features, four types of antifreeze proteins (AFPs; type I, II, III, and IV) were characterized from teleosts as well as antifreeze glycoproteins (AFGPs) (Lee et al., 2013). These proteins bind to the ice surface to prevent the growth of ice crystals by decreasing the freezing temperature. Generation of freeze-tolerant transgenic salmon or other species via the introduction of an AFP gene could greatly improve fish farming in northern latitudes.

The idea of producing transgenic fish with cold tolerance was actually motivated by the possibility of culturing Atlantic salmon along the East coast of Canada (Gómez, 2018). The Atlantic salmon is incapable to survive in sub-zero seawater temperature due to the lack of any of these AFGPs or AFPs gene(s) (Hew et al., 1995). This inability cause one of the major problems of sea cage farming in the northern Atlantic coast severely limiting the selection of suitable sites for operation. To solve this problem, winter flounder type I AFPs that have two isoforms, liver-type, and skin-type, could be good candidates for gene transfer. The former type mainly is produced in the liver as precursor proteins (preproAFPs) that need to be further processed while the latter type is produced in peripheral tissues as intracellular, mature AFPs (Hew et al., 1999). The AFP gene for type I AFP from winter flounder were inserted into Atlantic salmon genome under its promoter and expressed at a level of 0.1–50 µg/ml (Shears, 1991). Another study generated a stable transgenic line of Atlantic salmon by incorporating one copy of the winter flounder liver-type AFP gene into the genome of Atlantic salmon and the expression of the transgene was stable (approximately 250 µg/ml) up to the F3 generation (Hew et al., 1999). ProAFP was expressed only in the liver and display seasonal variations similar to those in winter flounder.

Figure 1. AquaBounty Technologies farm in Albany, Indiana established as first commercial-scale production facility of AquAdavantage Salmon.

The antifreeze activity was found in the sera of F3 offspring despite the lack of necessary processing enzymes to process proAFP precursor into a mature protein, but low-level production of AFP remained a problem (Hew et al., 1999).

Similar approaches were also conducted on goldfish by integrating the type III AFP gene from ocean pout encoding a mature protein into its genome (Wang et al., 1995). In this model, a mature AFP was produced in F1 and F2 offspring, and the transgenic goldfish showed better tolerance to cold water compared with the control group. Likewise, nile tilapia become resistant to temperatures as low as 13°C by injection or oral administration of AFP to juveniles or adults (Wu et al., 1998). This suggests that transgenic tropic species could be farmed in cold areas by the integration of AFP into their genome from other species, but low-level production of AFP decreases the efficiency of this approach. Whilst the expression level of AFP in winter flounder is generally approximately 10–20 mg/ml, all transgenic fish for AFP only express in the µg/ml range (Zbikowska, 2003). An increase in the copy number of the transgene or the use of constructs with other AFPs could increase expression and help to enhance freeze-resistance in farm fish.

Disease resistance

One of the promising application areas of gene transfer in aquaculture is the development of disease resistance. In aquaculture, diseases are an important problem disrupting animal welfare and leading to great economic losses. Transgenic fish with increased disease resistance could improve the profitability, production, efficiency, and welfare of the cultured fish (Dunham, 2009).

One approach to increase resistance against bacterial pathogens is to transfer antibacterial peptide genes. This approach was applied to Channel catfish (*Ictalarus punctatus*) by the transfer of a DNA construct containing a lytic peptide, cecropin B, driven by a cytomegalovirus (CMV) promoter and transgenic fishes showed two and four-fold increases in resistance against *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively (Dunham et al., 2002). Also, no difference in growth rate was observed between the transgenic and non-transgenic siblings. Another example of increasing bacterial resistance through transgenesis is the transfer of cecropin genes to medaka (*Oryzias latipes*) using various DNA constructs (Sarmasik et al., 2002). At the F2 generation, transgenic medaka from different families were challenged with *Psuedomonas fluorescens* and *Vibrio anguillarum,* and then transgenic lines showed 0–10% and 10–30% cumulative mortality, respectively. The enhanced disease resistance showed a difference between transgenic families, which means family variation could be important due to differences in the genetic background (Dunham, 2009). This emphasizes combining gene transfer with selection to provide maximum genetic gain from the gene transfer. Besides antibacterial peptides, other proteins with antimicrobial properties could be used to enhance disease resistance. B actin-human lactoferrin gene was transferred to grass carp (*Ctenopharyngodon idellus*) and then P1 individuals were more resistant to *Aeromonas* with increased phagocytic activity (Mao et al., 2004).

Another approach is to enhance the expression of a piscine lysozyme as an antibacterial agent against some fish pathogens. Yawaza et al. (2006) produced F2 transgenic zebrafish (*Danio rerio*) using a DNA construct including hen egg white (HEW) lysozyme gene and green fluorescence protein (GFP) gene driven by Japanese flounder (*Paralichthys olivaceus*) keratin promoter. Expression of both HEW lysozyme gene and GFP gene was detected in the liver and protein extracts from the liver of F2 transgenic fish showed 1.75 times higher lytic activity than in the controls. In a challenge experiment with *Flavobacterium columnare*, 65% of the F2 transgenic fish survived while 100% of the control fish were killed (Yawaza et al., 2006). Similarly, 60% of the F2 transgenic fish survived during a challenge test with *Edwardsiella tarda*, while 100% of the control fish died.

To prevent viral diseases in aquaculture, the common gene transfer approach is to use viral antisense RNAs or DNA-vaccines. However, the application of these techniques does not create exactly transgenic fish because these DNA/RNA constructs could not be passed to the next generation, instead, these techniques play a role in the gene expression of fish. Antisense RNA approach was applied to prevent infectious pancreatic necrosis virus pathogenicity by hammerhead ribozyme cleavage in vitro (Chen et al., 2000). The first DNA vaccine was produced against infectious hematopoietic necrosis using its the glycoprotein gene and tested on rainbow trout (Anderson et al., 1996). Since then, mono and multivalent DNA vaccines were applied to many fish species to protect against viral and bacterial pathogens (Kumar et al., 2008; Pereiro et al., 2012; Sun et al., 2012).

Transgenic fish in ecotoxicology

Transgenic fish, particularly transgenic zebrafish, have significant potential use in aquatic ecotoxicology as biosensors and models providing information on health effects of chemical exposure, but the use of transgenic fish in environmental toxicology is not common (Lee et al., 2014). Biosensor fish work in the principle of stimulation of specific genes, often enzymes or receptors, by certain chemicals/pollutants (Zbikowska, 2003). Transgenic fish could detect environmental pollutants in water and then induce a reporter gene driven by an element activated by low levels of pollutants. Transgenic fish lines developed to research contaminants and other environmental stressors include cadmium and copper toxicity by induction of heat-shock protein gene, oxidative stress via the induction of an electrophile-responsive element (EpRE), various organic chemicals interacting with the aryl hydrocarbon receptor-mediated toxicity, and estrogenicity (vitellogenin, choriogenins, estrogen

receptor-responsive elements) generally using either luciferase or GFP as reporter genes (Blechinger et al., 2002; Kusik et al., 2008; Lee et al, 2014; Mattingly et al., 2001; Petersen et al., 2013; Zeng et al., 2005). Transgenic biosensor fish were created by using the heat-shock protein (*hsp*) promoters, promotors of *hsp70* and *hsp27,* stimulated by various environmental stressors such as temperature and heavy metals (Lee et al., 2014). Transgenic zebrafish using *hsp 70* gene promoter to control eGFP (enhanced green fluorescent protein) as the reporter gene was exposed to cadmium and was sensitive at concentrations as low as 0.2 µM (22.5 µg/L) (Blechinger et al. 2002).

The promoter of the *cyp1a1* gene was used to control a GFP reporter gene for detection of exposure to organic chemicals in transgenic zebrafish and medaka (Hung et al. 2012; Kim et al. 2013; Lee et al, 2014; Ng & Gong 2013). *Cyp1a1* is a member of the cytochrome P450 superfamily and has a role in the oxidative metabolism of diverse organic substances including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Ma & Lu, 2007). Transgenic *Cyp1a - GFP* medaka embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*–dioxin (TCDD) for 24 hours and then GFP expression was detected in kidney, liver, and gut at an exposure concentration of only 0.005 nM (1.6 ng/L) (Ng & Gong, 2013). Also, this transgenic medaka expressed the GFP reporter gene in the liver and kidney when exposure to other PAHs including 3-methylcholanthrene (3-MC) and benzo[a] pyrene (BaP).

Furthermore, transgenic fish could be used for mutation assays to evaluate potential DNA damage after exposure to chemicals in aquatic environments. Transgenic medaka developed by Winn et al. (2000) harbors bacteriophage λ LIZ vector containing the *lacI* or *cII* bacterial gene as mutational targets. Also, mutation assays based on plasmid vectors were designed such as zebrafish carrying the pML4 plasmid vector fused to *rspL* gene (Amanuma et al., 2000). Thanks to the valuable features of fish as test organisms, transgenic fish could make important contributions to ecotoxicology studies.

Transgenic fish in Ornamental Fisheries

Ornamental aquaculture is a growing commercial sector with more than 4500 freshwater species and 1450 marine species traded worldwide (Stevens et al., 2017). Ornamental fish trade is a significant source of income for a lot of countries including Singapore, Malaysia, Thailand, and Indonesia while USA is the largest importer of Ornamental fish (Satam et al., 2018). Although accurate information about total value of the sector is lack, estimated total value of the sector varies between U.S. $800 million and $30 billion annually and the amount of traded fish is considered to be between 350 million and 1.5 billion live fishes (Stevens et al., 2017). Development of species with new features by transgenesis could contribute to the growth of this sector presenting customers with new options.

Transgenesis is applied on ornamental fish species to produce transgenic fish with different color forms. Color genes showed stable expression in zebrafish embryos injected with GFP expression constructs under the control of the zebrafish muscle-specific promoter of the *myosin light polypeptide 2* (*mylz2*) gene (Ju et al., 2003). Then fluorescent white skirt tetra (*Gymnocorymbus ternetzi*), medaka, and farmed rohu carp were successfully produced using the zebrafish *mylz2* promoter (Mohanta et al., 2014; Pan et al., 2008; Zeng et al., 2005). 'GloFish' with six attractive fluorescent color combinations, including Starfire red, cosmic blue, electric green, galactic purple, sunburst orange and moonrise pink have been already presented to the market as commercial value-added aquarium fishes by transgenesis (Figure 2) (Vick et al. 2012).

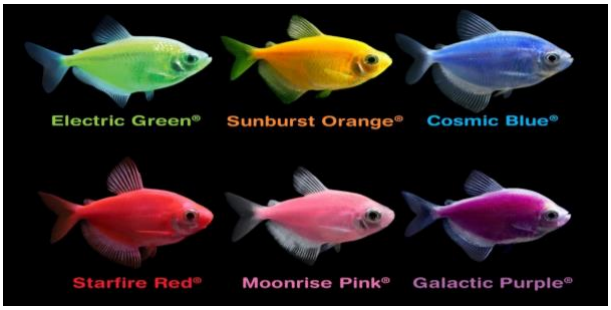

Figure 2. 'GloFish' with six attractive fluorescent color combinations, including Starfire red, cosmic blue, electric green, galactic purple, sunburst orange and moonrise pink as ornamental transgenic fish (Spectrum Brand Pet, 2020).

Gene Delivery Techniques in Fish

Microinjection

Microinjection of DNA into eggs or embryos at the one-cell stage is the most commonly applied gene transfer method in aquaculture (Pitkänen et al., 1999b; Cheers and Ettensohn, 2004). This technique was first used on goldfish for injection of foreign DNA into embryos and then applied on a variety of fish species such as common carp, atlantic salmon, medaka, tilapia, rainbow trout, and zebrafish to improve traits by gene transfer (Dunham et al., 1987; Hew et al., 1992; Ozato et al., 1986; Penman et al., 1991; Xu et al., 2008; Rahman et al., 1998; Rasal et al., 2016; Zhu et al., 1985).

Characteristics of eggs affect the application of microinjection and gene transfer efficiency. Fish eggs could have tough and opaque chorion which prevents insertion of glass micropipettes leading to low gene transfer efficiency rate (Dunham et al., 1987; Sin et al., 1997). Also, transgene could be injected into the cytoplasm of the egg because the nuclei of eggs could be small and hard to visualize. Limitations related to tough and opaque chorion dealed with injection into oocyte nuclei after making a hole in salmonids called two-step injection (Rasal et al., 2016). Eggs could be dechorionated manually or using trypsin or pronase to ease the insertion of pippets. Injection pipettes are chosen depending on the egg size of species to prevent

mortality because of mechanical damage on fertilized eggs (Tonelli et al., 2017a). In order to enhance the efficiency of the selection of transgenics, genetic markers could be co-injected with the transgene to monitor zygotes as using of GFP as a marker in zebrafish (Peters et al., 1995).

Beyond problems related to characteristics of eggs, the microinjection method is time-consuming since microinjection of a transgene is operated on only one embryo after another (Singh et al., 2019). It even requires a great deal of technical skill and comparatively expensive types of equipment including micromanipulators and microinjectors. Despite these mentioned limitations, microinjection is a favored technique for gene transfer with a success rate ranging from 10% - 70% in fishes (Powers et al., 1991).

Electroporation

Electroporation, the use of high-voltage electric shocks to introduce DNA into cells, is a procedure that is popular for introducing foreign genetic material into the cells of many different organisms (Potter and Heller, 2018). The standard protocol for all organisms involves cells being suspended in an appropriate, conducting buffer with the foreign gene being transfected, and then high-voltage electric shocks are used to make the cells more porous, allowing the introduction of the foreign gene into the cell. Transfected cells are then diluted and initially cultured in a non-selective medium. Afterward, appropriate selection is added, and cells are then separated and assessed for the introduction of the transgene (Potter and Heller, 2018).

Electroporation can be used to introduce transgenes in aquaculture. Either by inserting the transgene into spermatozoa (Celebi et al., 2003) or oocytes (Grabarek et al., 2002) before fertilization or by direct insertion into fertilized embryos (Kera, Agerwala and Horne, 2010). These have been applied with success *in vivo* results in salmon with observed mosaicism of the fish (Sin et al., 2000), shrimp (Arenal et al., 2000), nile tilapia (Lin, Chang and Chen, 2016), and medaka with changes being transferred through the germline (Hostetler, Peck and Muir, 2003).

The success of electroporation has shown to be extremely influential for zebrafish developmental biology studies. One example involves introducing a CRISPR/Cas9 plasmid to knockdown the gene *Mctp2p* to characterize its effect on neuronal and muscular development in zebrafish embryos (Espino-Saldaña et al., 2020). Electroporation is a diverse method with many potential applications for the development of transgenic fish.

Transgenesis Tools

Transposon Vectors

Transposable elements are a valuable tool to integrate genes into chromosomes to provide new traits. A transposon system usually contains a transgene sequence flanked by transposon-inverted repeats and the transposase-coding sequence (Tonelli et al., 2017a). *Sleeping Beauty*, *Tol2*, and *piggyBac* are commonly applied transposons for fish.

Transposon systems first were used on fish when a *Caenorhabditis elegans* transposon (*Tc3* element from the Tc1/mariner family) system was injected into one-cell-stage eggs to integrate GFP into the zebrafish genome (Raz et al., 1997; Tonelli et al., 2017a). *Tol2* element, derived from the medaka genome, was also used on zebrafish to deliver various genes (CFP/YFP/RFP or Gal4 cassettes) and this study provided new protocols to rapidly apply *Tol2* mediated zebrafish transgenesis (Bussmann & Schulte-Merker, 2011). Application of *Sleeping Beauty* transposon on zebrafish to transfer a blue-shifted GFP variant and a red fluorescent gene in a tissue-specific manner enhanced the transgenesis and expression rate sixfold (from 5 to 31%) compared to standard, plasmid injection-based transgenesis methods (Davidson et al., 2003). Applicability of the transposon *piggyBac* for transgenesis was showed on goldfish and loach (Hu et al., 2012).

Transposon vectors have a few advantages for transgenesis in fish compared to plasmid and viral vectors. Transposon vectors provide the insertion of a single, defined DNA sequence into the genome without absolute size restrictions instead of a multi-copy of sequence observed use of plasmid vectors (Hackett et al., 2004). However, possible active copies of the corresponding transposases might prevent insertion and enhanced stability of transgenes in fish such as salmonids (Tafalla et al., 2006).

Viral Vectors

Infection by viruses is a well-established gene delivery tool still used for transgenesis due to efficient integration and usually single-copy insertion of genetic material into the host genome. When retroviruses and lentiviruses are internalized into the target cell, their genetic material (RNA) is transformed to DNA, thereby their genetic information is inserted into the host genome (Tonelli et al., 2017a). After transduction efficiency of pseudotyped viruses was displayed on zebrafish in 1994, this method was applied on various fish species including nile tilapia and live-bearing fish (*Poeciliposis lucida*) to integrate transgene into the fish genome (Lin et al., 1994; Sarmasik et al., 2001; Tonelli et al., 2017b).

In order to improve this method for transgenesis in aquaculture, a number of viral gene delivery studies were conducted on zebrafish and medaka due to their transparent embryos (Tonelli et al., 2017a). Zebrafish cells were infected by vesicular stomatitis virus (VSV)-glycoprotein envelope including a genome obtained from the Moloney murine leukemia virus (MLV) and studies showed the degree of transduction efficiency expanded as the titer elevated and transmittable insertions could be enhanced in zebrafish by selecting virus-producer cell lines (Chen et al., 2002; Gaiano et al.,

1996; Lin et al., 1994). Also, transgenic zebrafish could be produced by applying viral gene delivery into the sperm culture and then performing in vitro fertilization (Kurita et al., 2004). Baculovirus system as that used on zebrafish could be used as an alternative to retrovirus and lentivirus because they provide increased control of the transduced region and exact determination of gene expression time using various promoters on DNA constructs (Wagle & Jesuthasan, 2003).

Despite the higher transduction efficiency of gene delivery by viral vectors, this method has some important disadvantages. Transgenes transferred by a viral vector could show unstable expression or even complete silencing of the transgene (Rasal et al., 2016). The reason for silencing of the transgene is that activation of gene repression machinery in the host cells through the promoter and enhancer sequences of the retroviral long-terminal-repeats (LTRs), followed by hypermethylation of the viral promoter sequences by *de novo* DNA methylation (Jahner and Jaenisch, 1985). Owing to the small size of viral vectors, generally, 10 kb transgene could be package into viral vectors, which restrict the transfer of larger genes by this method (Robl et al. 2007). Also, infection of early embryos usually shows a delay in transgene integration, thus producing mosaic animals that is because the breakdown of the nuclear membrane during mitosis is necessary for infection by retroviruses (Robl et al. 2007). Even though limitations of this method could be solved, the public may not accept transgenic fish as food produced by an integration of viral sequences because of biosafety and ethical issues (Rasal et al., 2016).

Tools for site-specific integration

In contrast to transgenesis, which involves the transfer of a gene from one organism to another, genome editing allows specific, targeted, and often minor changes to the genome of the species of interest. Three methods have been predominantly utilized to conduct genome editing. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR).

The earliest developed programable method of gene editing involves utilising a group of nucleases called zinc-finger nucleases (ZFNs). ZFNs are hybrids between a nonspecific DNA-cleavage domain and a DNA-binding domain composed of Cys_2His_2 zinc fingers. These nucleases have been used to stimulate homologous recombination of DNA, allowing the introduction of mutations (Bibikova et al., 2001). ZFNs have increased the efficiency of introducing foreign genetic material to the DNA of higher eukaryotes from approximately 1 for each 10^6 cells treated to nearly 100% of all cells treated. Gene editing is conducted by co-injecting to nuclease and DNA into the cells. ZFNs then cut specific restriction sites within the cell DNA and the introduced DNA is introduced via homologous overhangs, allow specific, site-directed mutagenesis (Bibikova et al., 2001). This technique has been used to introduce targeted mutations *in vivo* in many species including *Drosophila* (Bibikova et al., 2002) and zebrafish with mutations being carried through the germline for zebrafish (Foley et al., 2010). More recently, ZFNs have been applied *in vivo* to species of interest to aquaculture such as editing the luteinising hormone in channel catfish to create sterile fry (Qin et al., 2016) Notable limitations of gene editing through use of ZFNs include the need for specific restriction sites to be present within the gene of interest as well as two restrictions sites being located within 6 to 18 bp of each other (Bibikova et al., 2001). This restricts the capacity for developing point mutations and small edits within the genome using this technique and necessitates the application of other techniques to achieve these changes.

Transcription activator-like effector nuclease (TALENs) was later developed after the discovery of the transcription activator-like (TAL) family of proteins in the plant pathogenic bacteria *Xanthamonas (Boch and Bonas, 2010).* TALENs could be used for genome editing by inducing double-strand breaks (DSB), which activate repair mechanisms of cells (Joung & Sander, 2012). Non-homologous end joining (NHEJ) ligates DNA from either side of a double-strand break where there is little, or no sequence overlap for annealing. This induces errors in the genome via indels (insertion or deletion), or chromosomal rearrangement, resulting in a non-functional gene (Miller et al., 2011). TALENs has successfully been applied *in vivo* to edit the genome of aquaculture species including editing oestrogen production in nile tilapia (Li et al., 2013). A major limitation to the application of TALENs in aquaculture is that the mechanism of NHEJ only ablates genes and cannot create point mutations (Miller et al., 2011). This restricts gene editing to those genes where loss of function is beneficial while other methods of gene editing will be required where change or increase in function is required.

The most novel method of gene editing methods is CRISPR which uses the Cas family of proteins to introduce breakage to the DNA of the target organism allowing the introduction of genetic edits (Jinek et al., 2012). CRISPR and accompanying Cas proteins constitute an adaptive antiviral immune system in bacteria and archaea (Barrangou, 2015). The CRISPR defense system allows bacteria and archaea to recognize specific sequences and degrade them to prevent viral infection. CRISPR-Cas immune responses proceed in three stages: adaptation, where pieces of DNA are sampled from the invasive genetic material and are acquired into CRISPR loci for the purposes of immunity and immune memory, expression through transcription and processing of interfering CRISPR RNAs (crRNA), and interference through Cas directed cleavage of the invasive genetic material.

A subset of the CRISPR immune system response which utilizes mature crRNA base-paired to trans-

activating tracrRNA to form an RNA structure that directs CRISPR-associated protein Cas9 has been exploited for the purposes of gene editing (Jinek et al., 2012). The CRISPR/Cas9 gene-editing system creates site-specific double-stranded breaks in target DNA. Genes are edited by incorporating a target sequence with a neighbouring protospacer adjacent motif (PAM) specific to Cas9 into the guide RNA (gRNA) of the Cas9 vector. The Cas9 vector is then inserted into the target cells where it encodes for the gRNA and the Cas9 protein. The gRNA directs the Cas9 to the target DNA where the Cas9 creates a double-stranded break at the PAM site and the cells naturally repair the DNA using NHEJ. This mechanism is often inaccurate, leading to random insertions and deletions, and causing frameshifts in the target gene, which can knock out the entire gene. More targeted mutagenesis can be achieved by utilizing homology-directed repair (HDR). This process requires a homology-containing donor DNA sequence to be coinserted with the Cas9 vector to facilitate repair (Zhang et al., 2014). This sequence can contain specific mutations which will then be incorporated into the modified DNA as it repairs, creating potentially "error-free" mutagenesis.

Multiple gRNAs can also be incorporated into a single CRISPR/Cas9 vector, inducing multiple mutations within a genome or a single gene (Sakuma et al., 2014). This multiplexing system has been demonstrated in mammalian genomes as well as those of zebrafish, drosophila, *Caenorhabditis elegans* and bacteria (Zhang et al., 2014). However, CRISPR/Cas9 can also risk cleavage of highly homologous sites other than those intended, creating off-target mutations, and is entirely dependent on the location of a PAM site. This makes the selection of the gRNA sequence extremely important to minimize these risks.

CRISPR editing has successfully been applied *in vivo* to many species including those relevant to aquaculture research (Table 1). These include modification of immune function in rohu carp (Chakrapani et al., 2016) and growth in channel catfish (Khalil et al., 2017) and rainbow trout (Cleveland et al., 2018) and the pacific oyster (*Crassostrea gigas*) (Yu et al., 2019) with germline transmission of these gene edits being observed in channel catfish (Khalil et al., 2017).

Conclusion

Classical genetic studies were used to improve fish stocks providing valuable information about genetic traits. Nevertheless, scientists started to engineer a particular genetic trait in a directed way with the discovery of recombinant DNA technology and the development of gene transfer techniques. Therefore, transgenesis for enhancement of traits in a directed fashion started a new era in aquaculture. Transgenic fish have many significant biotechnological applications in several fields including aquaculture. Transgenic fish have been generated with enhanced growth rate, cold tolerance, and disease resistance for aquaculture. Also, transgenic fish have been developed for environmental monitoring and ornamental fisheries. Microinjection was used commonly as a gene delivery method despite its some disadvantages. Viral vectors or transposons are efficient transgenesis techniques, but DNA sequence randomly integrates into the genome that might disrupt a functional gene in the host using these techniques. This problem could be solved by using tools such as ZFNs, TALENs, CRISPR/Cas to achieve site-specific integration. These tools have successfully been applied to improve traits of fish stocks, but they have important disadvantages like off-target effect. Following biotechnological advances, new techniques with the increased efficiency and effectiveness will be proposed to generate transgenic fish solving problems related to current methods.

Besides technical problems, environmental risk and human safety aspects of transgenic fish should be considered. Environmental risk could be mitigated using inland farms and sterile animals as AquAdvantage salmon. Food-safety issues are dealing with regulatory agencies, but the fate of transgenic fish as food will be determined by consumers in the long term. While research on the generation of transgenic fish continues, consumers could be informed by education and campaign on technology for transgenic fish production to reduce their concerns.

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Table 1. *In vivo* CRISPR editing of organisms relevant to aquaculture studies

Species	Trait of interest	Method of introduction	NHEJ/HDR	Germline transmission	References
Labeo rohita	Immune fuction	Microinjection	HDR	No	(Chakrapani et al., 2016)
Ictalurus punctatus	Growth	Microinjection	NHEJ	Yes	(Khalil et al., 2017)
Crassostrea gigas	Growth	Microinjection	NHEJ	No	(Yu et al., 2019)
Pagrus major	Growth	Microinjection	NHEJ	No	(Kishimoto et al., 2018)
Salmo salar	Sterility	Microinjection	NHEJ	No	(Wargelius et al., 2016)
Oncorhynchus mykiss	Growth	Microinjection	NHEJ	No	(Cleveland et al., 2018)
Exopalaemon carinicauda	Molting	Microinjection	HDR	Yes	(Gui et al., 2016)
Oreochromis niloticus	Reproduction	Microinjection	NHEJ	Yes	(Li et al., 2014)

(*Litopenaeus schmitti*) by electroporation of single-cell embryos and injection of naked DNA into adult muscle. *Biotecnologia Aplicada*, *17*(4), 247–250.

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