Altering the Fatty Acid Biosynthetic Pathway with Multiple Transgenes to Elevate Omega-3 Fatty Acids Production in Channel Catfish (*Ictalurus punctatus*)

by

De Xing

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Approved by

Rex Alan Dunham, Chair, Professor of School of Fisheries, Aquaculture and Aquatic Sciences
Charles (Yiwu) Chen, Professor of Crop, Soil and Environmental Sciences
Ian Anthony Ernest Butts, Associate Professor of Fisheries, Aquaculture and Aquatic Sciences
Anthony G. Moss, Associate Professor of Biological Sciences

Abstract

Omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), play a very important role in human health. The channel catfish *(Ictalurus punctatus*) is one of leading freshwater aquaculture species in the US, but it has low levels of EPA and DHA compared to some fish, such as salmon. The biosynthesis of EPA and DHA in fish requires desaturation and elongation procedures. Elongase *elovl2* gene and desaturase Δ4 *fad*, Δ5 *fad*, Δ6 *fad*, *fat-1* (Δ15) and *fat-2* (Δ12) are all important genes limiting EPA and DNA biosynthesis pathway. To improve EPA and DHA content, we generated transgenic channel catfish carrying multiple elongase and desaturase transgenes, using several CRISPR/Cas9 strategies.

Channel catfish carrying masu salmon (*Oncorhynchus masou*) *elovl2* transgene were generated with random integration, CRISPR/Cas9 HDR (homology-directed repair)-mediated knockin (KI) and 2H2OP (two-hit by gRNA and two oligos with a targeting plasmid) methods. Integration rate of these three methods were 27.3%, 19% and 37.5%. DHA content of muscle increased by 20.7% - 62% in transgenic P1 fish produced by CRISPR/Cas9 technology compared to the non-transgenic fish.

To elevate KI efficacy and achieve multiple genes KIs in transgenic breeding program, we introduced a new strategy which enables transgene integration with numerous sites of genome by targeting long repeated sequence (LRS). Using this simple strategy, we successfully generated transgenic fish carrying masu salmon *elovl2* gene and rabbitfish (*Siganus canaliculatus*) Δ4 *fad* and Δ6 *fad* genes, and achieved robust KI of *elovl2* and Δ6 *fad* genes at multiple sites of LRS1 and LRS3, respectively, at one generation.

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Table of Contents

Abstract ii

Acknowledgments iii

List of Tables vi

List of Figures viii

List of Abbreviations xi

Chapter 1 Introduction 1

References 8

Chapter 2 CRISPR/Cas9- mediate knock-in method can improve the expression and effect of transgene in P1 generation of channel catfish (*Ictalurus punctatus)* 16

2.1 Abstract 16

2.2 Introduction 17

2.3 Materials and Methods 19

2.4 Results 24

2.5 Discussion 27

2.6 Conclusion 30

References 30

Chapter 3 CRISPR/Cas9-mediated transgenesis of the masu salmon (*Oncorhynchus masou*) *elovl2* gene improves n-3 fatty acid content in channel catfish (*Ictalurus punctatus)* 36

3.1 Abstract 36

3.2 Introduction 37

3.3 Methods 39

3.4 Results 45

3.5 Discussion 50

References 53

Chapter 4 A new strategy for increasing knock-in efficiency: multiple elongase and desaturase transgene knock-in by targeting at long repeated sequences in the channel catfish (*Ictalurus punctatus*) genome 60

4.1 Abstract 60

4.2 Introduction 61

4.3 Materials and Methods 63

4.4 Results 68

4.5 Discussion 75

References 78

Appendix A 85

List of Tables

Table 1 Primer sequences for identification of the transgenic channel catfish (*Ictalurus punctatus*) carrying masu salmon (*Oncorhyncus masou*) *elovl2* (*OmElovl2*) gene and for quantification of gene expression of transgene 22

Table 2 Table 2. Results of electroporation and screening of masu salmon, *Oncorhynchus masou*, *elovl2* transgene in putative transgenic channel catfish, *Ictalurus punctatus* 24

Table 3 Fatty acid compositions in muscle of P1 masu salmon, *Oncorhynchus masou*, *elovl2* (*OmElovl2*) transgenic channel catfish, *Ictalurus punctatus* produced by CRISPR/Cas9 technology and random integration, and their full-sibling non-transgenic controls that were grown in aquaria 27

Table 4 Primers used for CRISPR/Cas9 knockin and mutation screening for masu salmon, *Oncorhynchus masou*, *elovl2* transgene and the luteinizing hormone (*LH*) gene target site in putative transgenic channel catfish, *Ictalurus punctatus* 40

Table 5 Potential off-targets by prediction using online tool Cas-OFFinder (http://www.rgenome.net/cas-offinder/) (Bae et al., 2014) when using CRISPR/Cas9 to knockin the common carp β-actin-masu salmon, *Oncorhynchus masou*, elovl2 transgene for channel catfish, *Ictalurus punctatus* with luteinizing hormone gene as the target site 41

Table 6 Fatty acid compositions (% of total fatty acids) of muscle from P1 common carp β-actin-masu salmon, *Oncorhynchus masou*, *elovl2* transgenic channel catfish, *Ictalurus punctatus* and their full-sibling non-transgenic controls that were grown in aquaria 48

Table 7 Primers used for identification, gene expression and junctions detection of masu salmon (*Oncorhynchus masou*) elovl2 gene, rabbitfish (*Siganus canaliculatus*) Δ4 fad and Δ6 fad genes in transgenic channel catfish, *Ictalurus punctatus* 69

Table 8 Comparison of strategies for multiple genes KIs in channel catfish, *Ictalurus punctatus,* for hatching rate, fingerling survival rate, PCR positive (%) and on-target KI efficiency 71

Table 9 Percentage of single, double and triple transgene, masu salmon (*Oncorhynchus masou*) elovl2 gene, rabbitfish (*Siganus canaliculatus*) Δ4 fad and Δ6 fad genes, insertions for channel catfish (*Ictalurus punctatus*) targeting long repeated sequences (LRSs) 71

List of Figures

Figure 1 The biosynthetic pathway of n-3 PUFAs in animals 3

Figure 2 Schematic representation of producing mosaic transgenic channel catfish (*Ictalurus punctatus*) 18

Figure 3 The design and detection of elovl2 transgene in channel catfish, *Ictalurus punctatus*, using the CRISPR/Cas9 technology 20

Figure 4 RT-PCR analyses of masu salmon, *Oncorhynchus masou*, *elovl2* gene (*OmElovl2*) expression in different tissues of P1 transgenic channel catfish, *Ictalurus punctatus* 26

Figure 5 Strategy and molecular characterization of gene transfer of the common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgene integration into channel catfish, *Ictalurus punctatus* 40

Figure 6 Gene transfer and expression of the common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgene in channel catfish, *Ictalurus punctatus* grown in aquaria 45

Figure 7 Morphology of transgenic common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* and control channel catfish, *Ictalurus punctatus* 46

Figure 8 Schematics of HDR- (homology-directed repair-) and NHEJ- (non-homologous end joining-) mediated knock-ins (KIs) of three donor plasmids targeting three long repeated sequences (LRSs) in genome of channel catfish, *Ictalurus punctatus* 66

Figure 9 PCR detection of single, double and triple transgene, masu salmon *(Oncorhynchus masou) elovl2* gene, rabbitfish (*Siganus canaliculatus)* Δ4 *fad* and Δ6 *fad* genes, insertions for channel catfish (*Ictalurus punctatus*) targeting long repeated sequences (LRSs) 70

Figure 10 Knock-in (KI) of masu salmon (*Oncorhynchus masou*) elovl2 and rabbitfish (*Siganus canaliculatus*) Δ6 fad genes into long repeated sequences (LRS) of channel catfish (*Ictalurus punctatus*) genome 73

Figure 11 Detection of masu salmon *(Oncorhynchus masou) elovl2*, rabbitfish (*Siganus canaliculatus)* Δ4 and Δ6 transgene expression in triple transgenic channel catfish (*Ictalurus punctatus*) utilizing RT-PCR 74

Figure S1 Schematics of HDR- (homology-directed repair-) mediated KIs of three donor plasmids driven by common carp β actin promoter targeting three non-coding regions, respectively, in genome of channel catfish, *Ictalurus punctatus* 84

Figure S2 Schematics of ssODN-mediated end-joining KIs of masu salmon *(Oncorhynchus masou) elovl2* gene, rabbitfish (*Siganus canaliculatus)* Δ4 *fad* and Δ6 *fad* genes driven by common carp β actin targeting *LH* (luteinizing hormone), *FSH* (follicle-stimulating hormone) and *GnRH1* (gonadotropin-releasing hormone 1) in channel catfish, *Ictalurus punctatus* 85

Figure S3 Sequence at LRS1 after insertion of the UBI_masu_salmon_elovl2 donor plasmid with perfect HDR-mediated knock-in 86

Figure S4 Sequence at LRS1 after insertion of the UBI_masu_salmon_elovl2 donor plasmid with perfect NHEJ-mediated knock-in 88

Figure S5 Sequence at LRS2 after insertion of the UBI_rabbitfish_Δ4 donor plasmid with perfect HDR-mediated knock-in 91

Figure S6 Sequence at LRS2 after insertion of the UBI_rabbitfish_Δ4 donor plasmid with perfect NHEJ-mediated knock-in 94

Figure S7 Sequence at LRS3 after insertion of the UBI_rabbitfish_Δ6 donor plasmid with perfect HDR-mediated knock-in 97

Figure S8 Sequence at LRS3 after insertion of the UBI_rabbitfish_Δ6 donor plasmid with perfect NHEJ-mediated knock-in.. 100

List of Abbreviations

N-3	Omega-3
LC-PUFAs	Long-chain polyunsaturated fatty aicds
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
ZFN	Zinc finger nuclease
OmElovl2	Masu salmon (*Oncorhynchus masou*) *elovl2* gene
TALEN	Transcription activator-like effector nuclease
CRISPR	Cluster regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
LH	Luteinizing hormone
HDR	Homology-directed repair
NHEJ	Non-homologous end joining
FSH	follicle-stimulating
GnRH1	gonadotropin-releasing hormone 1

Chapter 1 Introduction

Omega-3 (n-3) fatty acids are polyunsaturated fatty acids (PUFAs) characterized by the presence a double carbon-carbon bond at third carbon atom (n-3 position) from the end of the carbon chain (Kapoor and Patil, 2011). Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are well known n-3 fatty acids, which modulate a number of cell functions in animals and humans (Calder, 2012). There are plenty of evidences revealed that n-3 fatty acids play an important role in brain development and function (Innis, 2007), might prevent or ameliorate cardiovascular disease (Breslow, 2006), autoimmune disorders (Fernandes et al., 2008), crohn disease (Chan et al., 2014), cancers of the breast, colon and prostate (Larsson et al., 2004), rheumatoid arthritis (James and Cleland, 1997) and so on. Governments of UK, France, New Zeeland and Australia, and health organizations, FAO/WHO and American Heart Association, recommend dietary intakes for 1.4-2.5g/d total n-3 PUFA with EPA and DHA ranging from 140-600 mg/d (Molendi-Coste et al., 2010).

As currently understood, the n-3 PUFAs biosynthetic pathway (as shown in Figure 1) in mammals consists of elongase and desaturase procedures. An initial desaturation of α-linolenic acid (ALA, 18:3n-3) by Δ6 desaturase to stearidonic acid (18:4n-3), followed by elongation by elongase gene to eicosatetraenoic acid (20:4n-3), Δ5 desaturation to EPA, elongation to docosapentaenoic acid (DPA, 22:5n-3) by elongase 2 and 5, another elongation to tetracosapentaenoic acid (24:5n-3) by elongase 2, then Δ6 desaturation to tetracosahexaenoic acid (THA, 24:6n-3), and finally the peroxisomal β-oxidation of THA to DHA (Metherel and Bazinet, 2019). It was demonstrated that Δ4 desaturase could convert EPA to DHA directly (Li et al., 2010). Thus, humans are able to obtain either EPA and DHA directly or the precursor

ALA and intermediates in the pathway. Currently, most dietary sources of n-3 fatty acids are oily marine fish including such as salmon, tuna, herring, sardines (Hashim et al., 2021). Over the past decades, Fish supplement continuously increased, over 158 million tones (89%) of total fishery and aquaculture production was used for direct human consumption in 2019. However, there was a reduction of non-food fish production since 1990s, with only 11% of total fish production destined mainly for fishmeal and fish oil, which used as feed ingredients for aquaculture and livestock feeds, in 2019 (FAO, 2021). With increasing global fish oil costs, present fish oil could not meet these demands any more (Tacon and Metian, 2008). In order to reduce dependence on fish oil, plant oils were used to replace fish oil in compound fish feeds. However, compared with animals, most plants like vegetables and crops lack EPA and DHA, due to the absence of essential enzymes for biosynthesis of EPA and DHA. Although flaxseed oils and some nuts contain high ALA, these sources are not usually consumed consistently or in large quantities. Previous studies revealed partial replacement of fish oil by vegetable oils in fish feed did not impact growth performance, but would reduce EPA and DHA contents of fish muscle (Nasopoulou and Zabetakis, 2012). Microalgae are the primary producers of EPA and DHA fatty acids that accumulate over the trophic levels in the marine food webs. But microalgae productivity is limited, and unable to be main source of n-3 fatty acids (Chauton et al., 2015).

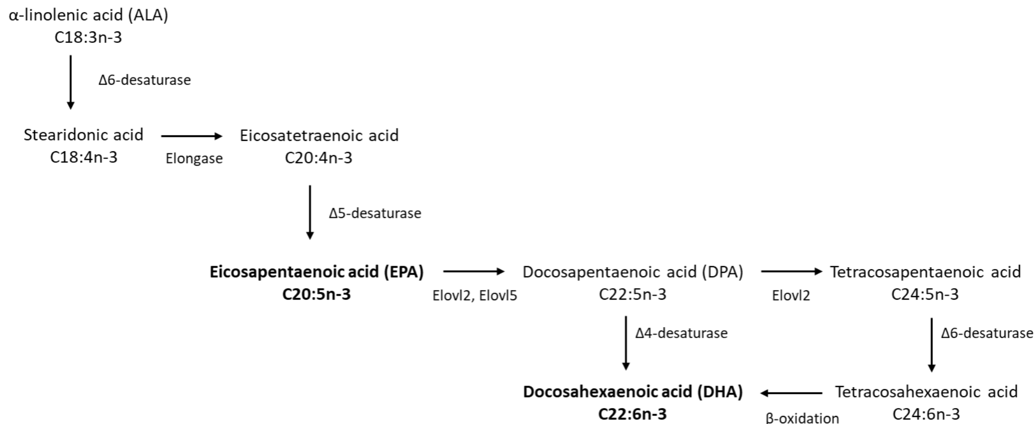

Figure 1. The biosynthetic pathway of n-3 PUFAs in animals

Catfish farming was and is largest aquaculture industry in United states of America, accounting for 75% of total domestic finfish aquaculture production. Currently, the catfish industry is a major contributor to the rural economies of the Southern states including Alabama, Arkansas, and Mississippi (Hegde et al., 2021). However, catfish production suffered a 64% reduction since 2003 (Hanson, 2019). The factors that caused the decline of the catfish industry included intense competition from imported products from Asia, increased feed and fuel costs, as well as fish disease control (Wagner et al., 2002); FAO 2011). However, foreign imports now have ~73% of the catfish market in the U.S. Strong competition from imports forced domestic farms to reduce price and profits. Thus, improving catfish production value is the best way to recapture this market share would have large economic implications for U.S. and rural communities while restoring the catfish industry to its peak production levels or more.

Channel catfish as freshwater fish are not n-3 fatty acids rich fish. Sum of EPA and DHA contents were only 0.75-4.4% (Hedrick et al., 2005; Li et al., 2009; Morris et al., 1995). If we could elevate n-3 fatty acids levels in channel catfish, we would not only make channel catfish fleets become more healthier production for customers, but also build a way to provide fish oil for aquafeed, which would increase value and price of channel catfish undoubtedly.

Transgenic fish research began almost 40 years ago (Maclean and Talwar, 1984; Zhu et al., 1985), which already had many potential applications in aquaculture. Transgenic Atlantic salmon (*Salmo salar*), coho salmon (*Oncorynchus kisutch*), tilapia (*Oreochromis niloticus*), channel catfish, common carp (*Cyprinus carpio*), rainbow-trout (*Oncorhynchus mykiss*) carrying foreign growth hormone genes were produced to enhance growth successfully (Chatakondi et al., 1995; Chen et al., 1993; Devlin et al., 1995; Devlin et al., 2001; Du et al., 1992; Dunham et al., 1987; Rahman et al., 1998). Antifreeze protein genes were transferred into goldfish (*Carassius auratus*) and Atlantic salmon to improve freeze resistance and cold tolerance (Shears, 1991; Wang et al., 1995). Additionally, transgenic channel catfish and rainbow trout expressing cecropin pipette exhibited disease resistance to fish bacterial pathogens (Chiou et al., 2014; Dunham et al., 2002). Zebrafish containing fluorescent protein cassette were generated by transgenesis for analysis of gene function and cell behavior (Chen et al., 2016). There also were studies referring to improve n-3 fatty acids by transgenesis. Transgenic nibe croaker (*Nibea mitsukurii*) carrying masu salmon (*Oncorhynchus masou*) *elovl2* transgene had higher DPA and lower EPA than non-transgenic fish (Kabeya et al., 2014). Expression of elongase-like gene from masu salmon in transgenic zebrafish improved both EPA and DHA content compared with non-transgenic fish (Alimuddin et al., 2008). Transgenic P1 common carp carrying masu salmon Δ5-desaturase-like gene contained higher DPA than controls (Cheng et al., 2014). Transgenic F1 channel catfish expressing masu salmon Δ5-desaturase-like gene showed 33% increase of total n-3 fatty acids. Fat-1 F1 transgenic showed great 4.8-fold and 1.9-fold increase in EPA and DHA, respectively (Zhang et al., 2019). Double transgenesis of fat-1 and fat-2 gene in zebrafish improved the 20:5n-3 and 22:6n-3 contents to 1.7- and 2.8-fold, respectively (Pang et al., 2014). Besides transgenic fish, transgenic mice and pig carrying fat-1 gene converted n-6 fatty acids to

n-3 fatty acids efficiently. However, these studies also clarified the fact that one or two foreign elongase or desaturase genes could not elevate n-3 fatty acids levels greatly since multiple limit the biosynthetic pathway. Therefore, multiple transgenes are required to elevate n-3 fatty acids level of channel catfish by transgenesis.

In the past, transgenic fish were produced by introducing foreign gene plasmids directly into fertilized eggs using microinjection or electroporation (Du et al., 1992; Zhang et al., 1990). With this early technology, foreign genes were integrated into host genome randomly, which might lead to low efficiency, high level of mosaicism, and increased mortality of transgenic embryos since foreign genes might interrupt important genes and their function in the host genome (Maclean and Laight, 2000). Additionally, many transgenes were strongly influenced by their sites of integration in the host chromosome in a positive or negative way. When transgene is inserted into close proximity to heterochromatin such as telomere and centromere regions. Its activity can become unstable, resulting in variations in expression level referred to as mosaicism or variegation (Eszterhas et al., 2002). Genomic DNA surrounding a well-expressed transgene favors the expression of another transgene when inserted into it, such as the ROSA26 locus in mouse (Strathdee et al., 2006). In addition, recent study showed that transgene near super-enhancer regions had higher transcript levels than near inaccessible regions (Lee et al., 2021). Certain transgenes are capable of expressing independently of their site of integration. The level of expression is directly correlated with the copy number of transgene (Peach and Velten, 1991). Bacterial artificial chromosome (BAC) transgenes copies positively correlated with gene expression in mice (Chandler et al., 2007). In some case, multiple copies of the transgenes with a head-to-tail manner may repress expression due to transcriptional interference (Strathdee et al.,

2006). However, conventional random integration was unable to control integration sites and copy numbers. Thus, better gene-editing tools were desirable to applied in transgenic research.

Zinc finger nucleases (ZFNs) directs gene editing events by linking the DNA-binding domain of a versatile class of transcription factors named zinc finger proteins (ZFPs) with the nuclease domain of the *FOK*I restriction enzyme (Urnov et al., 2010). ZFNs are considered as first “practical” DSB- assisted gene-editing tool that has been applied for introduce cleavages into the intended DNA sequences (Kalds et al., 2019). ZFNs technology was applied in studies of specific gene modifications (Qin et al., 2016; Santiago et al., 2008; Urnov et al., 2005). However, there is no applications of ZFNs reported in transgenesis as it is difficult to be designed.

Transcription activator-like effector nucleases (TALENs), like ZFNs, consist of DNA-binding motif (TALE effector repeats) and *FOK*I nuclease. TAL effectors, discovered in plant pathogens, contains 10-30 tandem repetitive sequences, each of which has ~35 amino acids to assist recognition specificity. TALENs are superior to ZFNs in terms of simple and straightforward design (Carlson et al., 2012). Most importantly, TALENs had high efficiency in inducing locus-specific knockout (KO) in medaka and zebrafish (Ansai et al., 2013; Bedell et al., 2012). Although short sequence knockin (KI) was achieved in zebrafish, rat and mouse (Bedell et al., 2012; Gagnon et al., 2014; Renaud et al., 2016), long transgene is not allowed to KI with TALENs.

Clustered regularly interspaced short palindromic repeats (CRISPR)- CRISPR-associated protein 9 (Cas9) gained rapid popularity these days due to its simplicity and high efficiency (Barrangou and Doudna, 2016). In this system, The RNA guided Cas9 protein recognizes a 5’-NGG-3’ PAM (protospacer-adjacent-motif) region and cut the specific DNA sequence, inducing

double strands break (DSB). Then three main pathways including non-homologous end-joining (NHEJ), homology-directed repair (HDR) and microhomology-mediated end-joining (MMEJ) would direct the repair the DSB, resulting in KO or KI (Fu et al., 2021). Currently, CRISPR/Cas9 has a wide number of applications for gene editing in fish (Wang et al., 2021). For example, generation of *myostain* gene KO to improve growth performance in red sea bream (*Pagrus major*) (Kishimoto et al., 2018; Ohama et al., 2020), channel catfish (Khalil et al., 2017), medaka (Yeh et al., 2017), blunt snout bream (*Megalobrama amblycephala*) (Sun et al., 2020), olive flounder (*Paralichthys olivaceus*) (Kim et al., 2019). KO *tyr* gene to result in pigmentation loss in zebrafish (Jao et al., 2013), large-scale loach (*Paramisgurnus dabryanus*) (Xu et al., 2019) and Atlantic salmon (Edvardsen et al., 2014). Additionally, mutations of *elovl2* (Datsomor et al., 2019b), Δ5 and Δ6 (Datsomor et al., 2019a), *AHR2* (Aluru et al., 2015), *gol* and *kctd10* (Zu et al., 2016) induced by CRISPR/Cas9 were used to study gene functions. However, The number of KI studies of fish are much less than KO. Reporter gene like GFP or RFP precisely integrated with genome of medaka (Gutierrez-Triana et al., 2018; Murakami et al., 2017) and zebrafish (Kimura et al., 2014; Wierson et al., 2019). Alligator cathelicidin gene KI was achieved channel catfish (Simora et al., 2020). Although CRISPR/Ca9 technology is superior than ZFNs and TALENs, High frequency of off-target effects and mosaicism are concerned, especially for gene therapeutic applications and breeding programs. Therefore, some strategies such as non-viral delivery (Glass et al., 2018), rational design of sgRNA (Xie et al., 2018), chemical modification (Ryan et al., 2018), utilization engineered Cas9 variants (Aquino-Jarquin, 2021) to reduce off-target effects of CRISPR/Cas9 system.

At present, The U.S. Food ang Drug administration (FDA) has approved GH transgenic salmon to be sold in the United States. And Japan has granted the permissions to sale two

CRISPR gene editing fish: a tiger puffer and a red sea bream. I believe there will be more gene editing or transgenic fish approved for sale in market in the future with CRISPR technology development. Our long goal is producing transgenic channel catfish carrying multiple foreign elongase and desaturase genes to elevate EPA and DNA levels of channel catfish, make catfish even more healthier, and benefit the catfish industry and customers.

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Chapter 2 CRISPR/Cas9- mediate knock-in method can improve the expression and effect of transgene in P1 generation of channel catfish (*Ictalurus punctatus)*

2.1 Abstract

Transgenesis has a wide range of applications in fish breeding and generation of fish models. Previously, it was common to produce transgenic fish by transferring plasmid DNA into early embryos, resulting in random integration, but more precision, targeted integration is possible with CRISPR/Cas9 technology. Channel catfish (*Ictalurus punctatus*) is an economically important farmed fish in the United States. To make channel catfish an even richer source of nutrients, we produced P1 fish carrying masu salmon (*Oncorhynchus masou*) *elovl2* (*OmElovl2*) transgene to increase the content of omega-3 (n-3) fatty acids with CRISPR/Cas9-mediated knock-in and random integration methods. Mosaicism, transgene expression and fatty acids contents were determined. Integration rate, 19%, generated by CRISPR/Cas9 method at seven months was not higher than random integration (27.3%) in the channel catfish. However, 13 out of 15 tissues were verified to carry the transgene from three positive P1 fish produced by CRISPR/Cas9 technology. Only five of 15 tissues carrying transgene were detected in three positive P1 fish produced by random integration. Additionally, RT-PCR and fatty acids analysis revealed that CRISPR/Cas9 P1 fish had strong *OmElovl2* transgene expression in most tissues and 20.7% higher DHA than their controls, while randomly integrated P1 fish did not have detectable *OmElovl2* expression in any of five tissues detected. There were no significant differences for any fatty acids between transgenic fish produced by random integration and their non-transgenic controls. CRISPR/Cas9 mediated knock-in technology efficiently reduced mosaicism, improved transgene expression and the biological effects of the foreign gene in P1 generation compared to the conventional random integration method. Therefore, transgenesis

based on CRISPR/Cas9 technology should benefit short breeding programs and improve applications of gene function studies.

2.2 Introduction

Transgenic fish research began almost 40 years ago (Maclean and Talwar, 1984; Zhu et al., 1985) with the goal of enhancing growth (Chen et al., 1993; Devlin et al., 2001; Dunham et al., 1987), freeze resistance (Shears, 1991) and disease resistance (Chiou et al., 2014) of aquaculture fish. Previous research showed that transgenic fish could be produced by introducing foreign gene plasmids into fertilized fish eggs using microinjection or electroporation (Du et al., 1992; Zhang et al., 1990).With this early technology, foreign genes were integrated into host genome randomly, which might lead to low efficiency, high level of mosaicism, and increased mortality of transgenic embryos since foreign genes might interrupt important genes and their function in the host genome (Maclean and Laight, 2000; Tonelli et al., 2017). To overcome these pitfalls, new technologies like *Tol2* transposons system, zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) have been applied to accomplish site-directed genomic integration of transgenes. However, these methods are complex, require the assembly of engineered proteins for the target sequence (Cong et al., 2013; Mali et al., 2013) and easily induce off-target cuts that limits their applications in transgenesis research (Tonelli et al., 2017). Therefore, clustered-regularly interspaced short palindromic repeats/CRISPR-associated Cas9 protein (CRISPR/Cas9) rapidly became a popular gene editing tool due to its versatility, cost-effectiveness and high efficacy. The CRISPR/Cas9-mediated knock-in can achieve highly precise and efficient insertion of a large DNA fragment in the genome by homology-independent double-strand break (DSB) repair pathways that have been demonstrated in zebrafish (*Danio rerio*) (Auer et al., 2014), rat (*Rattus norvegicus*) (Ma et al., 2014; Yoshimi et al., 2016), mouse

(*Mus musculus*) (Jang et al., 2018), Atlantic salmon (*Salmo salar* L.) (Straume et al., 2020) and channel catfish (*Ictalurus punctatus*) (Simora et al., 2020). Mosaicism is still common in P1 generation using CRISPR/Cas9 system since the translation of the introduced Cas9 DNA/mRNA into its active enzymatic form is likely delayed until after the first cell division (Figure 2) (Mehravar et al., 2019). It has been reported that direct Cas9 protein delivery in early stage embryonic development reduces the occurrence of mosaicism (Aida et al., 2015).

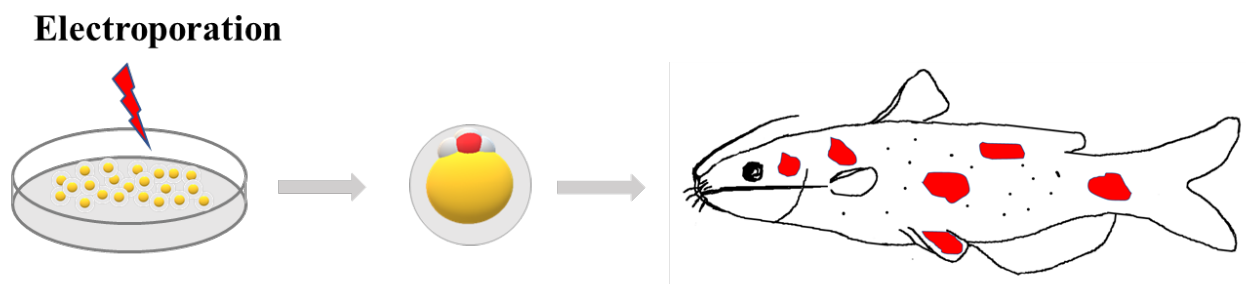

Figure 2. Schematic representation of producing mosaic transgenic channel catfish (*Ictalurus punctatus*). Only plasmid or the mixture of plasmid and CRISPR/Cas9 system are electroporated into fertilized eggs, but integration happens in one cell (red) during the 4-cells stage, resulting in mosaic transgenic fish.

Omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFAs), including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are linked to prevention of inflammatory and cardiovascular diseases (Dyall, 2015). Compared to EPA, DHA has specifically contributed to the development of brain, retinal and neural tissues in fetuses and young children (Fraeye et al., 2012; Jordan, 2010). However, n-3 LC-PUFAs are found in only a few foods, primarily oily fish, select nuts and seeds (Jordan, 2010). Channel catfish is the primary freshwater aquaculture species in the United States of America, but contains relatively low levels of n-3 LC-PUFAs relative to marine fish (Robinson et al., 2001). Biosynthesis of LC-PUFAs in vertebrates involves alternating steps of desaturation and elongation (Sprecher, 2000). *Elovl2* has greatest activity in elongation of C20 and C22 (Morais et al., 2009). Knockout of *elovl2* in Atlantic salmon can reduce levels of DHA and increase EPA (Datsomor et al., 2019). In

contrast, transgenic nibe croaker (*Nibea mitsukurii*) carrying masu salmon (*Oncorhynchus masou*) *elovl2* (*OmElovl2*) transgene have higher DPA and lower EPA than non-transgenic fish (Kabeya et al., 2014). Expression of elongase-like gene from masu salmon in transgenic zebrafish improved both EPA and DHA content compared with non-transgenic fish (Alimuddin et al., 2008). Therefore, knock-in of *elovl2* isolated from masu salmon may modify the n-3 LC-PUFA biosynthetic pathway, increasing n-3 fatty acids content in channel catfish.

With development of CRISPR/Cas9 technology, more study of CRISPR/Cas9 methodology is recommended due to its precise knock-in compared to the random integration method (Tonelli et al., 2017), but no study has compared these two methods directly. In this study, we evaluated two types of transgenic fish produced by two different methods - random integration (only plasmid transfer) and CRISPR/Cas9 targeted insertion, aiming at developing transgenic channel catfish lines carrying actively expressing *OmElovl2* gene with highly positive biological effects that can be inherited by subsequent generations.

2.3 Materials and Methods

2.3.1 Construction of donor plasmid

The donor plasmid was constructed with masu salmon *elovl2* gene (NCBI Accession: KC847063) driven by carp β-actin promoter and followed by ocean pout antifreeze polyA terminator. The donor constructs were flanked by 0.8kb left and 0.8kb right homology arms derived from the non-coding region of channel catfish chromosome 1 (Figure 3A).

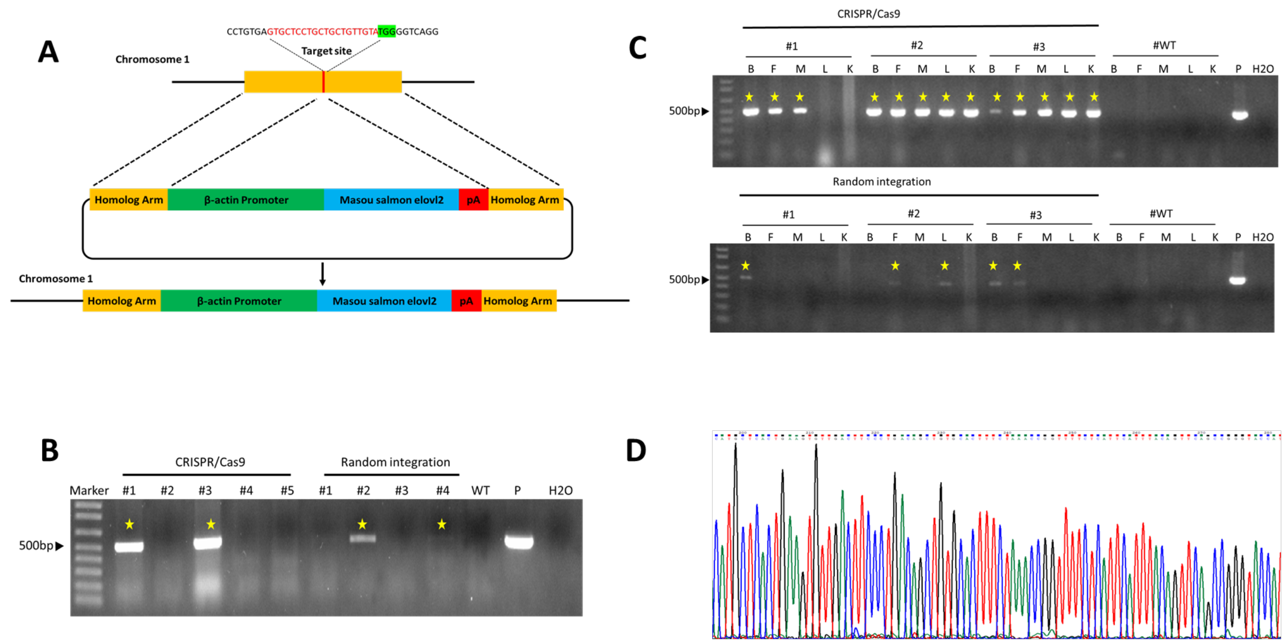

Figure 3. The design and detection of *elovl2* transgene in channel catfish, *Ictalurus punctatus*, using the CRISPR/Cas9 technology
(A) Construct to produce transgenic channel catfish, *Ictalurus punctatus*, carrying masu salmon, *Oncorhynchus masou*, *elovl2* gene (*OmElovl2*).
(B) PCR analysis of barbel and fin mixed samples of P1 transgenic fish produced by CRISPR/Cas9 and random integration. WT: wild type control. P: 20ng transgene template (positive control), H2O: water template (negative control).
(C) PCR analysis of different tissues in 3 P1 transgenic fish produced by CRISPR/Cas9 and random integration, respectively. The tissues are B: barbel; F: fin; M: muscle; L: liver; K: kidney. WT: wild type control. P: 20 ng transgene template (positive control), H2O: water template (negative control).
(D) Sequencing result of PCR product.

2.3.2 Design and preparation of sgRNA and CRISPR/Cas9 system

The CRISPR design online tool (https://zlab.bio/guide-design-resources) was used to design the small guide RNAs (sgRNAs) that targeted the channel catfish non-coding region of chromosome 1 (Figure 3A). Two double-stranded DNA oligos (Thermo Fisher Scientific, Waltham, MA), containing the T7 promoter and the 20-nt gene-specific target sequence and the sgRNA scaffold were annealed to generated the sgRNA templates using cloning free (PCR-based) method (Gagnon et al., 2014; Varshney et al., 2015) with modifications (Khalil et al., 2017). The sgRNA template was used to transcribe RNA by Maxiscript T7 Kit (Thermo Fisher

Scientific, Waltham, MA). The sgRNAs were purified using Zymo RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). The Cas9 protein was obtained from PNA BIO Inc. (Newbury Park, CA).

2.3.3 Ethical statement

Channel catfish were obtained from the Fish Genetic Research Unit, School of Fisheries, Aquaculture and Aquatic Science at Auburn University, Alabama 36849, USA. The research protocol followed all Standard Operating Procedures (SOP) approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University.

2.3.4 Brood stock spawning

Brood stock spawning was performed according to Qin et al. (2016) with modifications. Female fish were implanted with 100 μg/kg of luteinizing hormone releasing hormone analog (LHRHa) to induce ovulation, and eggs were hand-stripped into metal pie pans with grease. Sexually mature channel catfish males were euthanized and the testes were removed, crushed and macerated into saline (0.9% NaCl) to prepare sperm solution (Khalil et al., 2017).

2.3.5 Electroporation, incubation and rearing

Electroporation followed the standard procedures (Su, 2012). CRISPR/Cas9 and random integration method had almost the same electroporation procedures except that CRISPR/Cas9 system required sgRNA and Cas9 protein, but random integration didn't. In a brief, two drops of sperm solution were added to the 2 ml *OmElovl2* plasmid (50 ng/μL) saline solution. Around 6 μL sgRNA (300 ng/μL) and 6 μL Cas9 protein (1 mg/μL) for CRISPR/Cas9 method or 12μL 0.9% saline for random integration method were added into the solution, then completely mixed, and incubated at room temperature for 5 min. Then the mixture was poured into a 10-ml petri dish and diluted with 2 ml freshwater, followed by electroporation using a Baekon 2000

macromolecule transfer system (Baekon, Inc. Saratoga, CA) with parameters set at 6 kV, 2^7 pulses, 0.8s burst, four cycles, and 160 µs (Powers et al., 1992). One hundred and fifty eggs were fertilized with this sperm and incubated in freshwater for 60 min, then transferred into 10-ml petri dish to be electroporated again as described above. All electroporated fish were reared in Holtfreter's solution (Bart and Dunham, 1996) containing 10 ppm doxycycline at 27 °C until hatching. Then fry were transferred into a recirculating system with 2 fry/L density. Fry were first fed Aquamax fry powder (50% crude protein, 17% crude fat, 3% crude fiber and 12% ash) (Purina Animal Nutrition LLC, Shoreview, MN) four times a day for two months. Fingerlings were then fed Aquaxcel WW Fish Starter 4512 (45% crude protein, 12% crude fat, 3% crude fiber and 1% phosphorus) (Cargill Animal Nutrition, Minneapolis, MN) twice a day.

2.3.6 Sample collection and integration analysis

The mixed fin and barbel clip samples of seven-month-old fingerlings were extracted via proteinase K followed by protein precipitation as described by Kurita at el. (2004). Specific primers of *OmElovl2* were designed using NCBI primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). PCR products were electrophoresed on a 1% agarose gel to check bands (Figure 3B), then inserted into the vector of the TOPO TA Cloning Kit (Invitrogen) for sequencing and transformed into One Shot TOP10F chemically competent *E. coli* (Invitrogen). Colonies were sequenced by Genewiz (South Plainfield, NJ) (Figure 3D). Then the sequencing results were blasted with transgenes using MAFFT (version 7, https://mafft.cbrc.jp/alignment/server/) to identify positive fish.

Table 1. Primer sequences for identification of the transgenic channel catfish (*Ictalurus punctatus*) carrying masu salmon (*Oncorhyncus masou*) *elovl2* (*OmElovl2*) gene and for quantification of gene expression of transgene.

Primer	Sequence (5'-3')	Product size (bp)	Description
*OmElovl2*_F *OmElovl2*_R	CGAAATCCGTTCCTTTTTACTG CTGGCCTGTTCCTCATGTATTT	499	Masu salmon *elovl2* transgene detection

*OmElovl2*_RT_F3 *OmElovl2*_RT_R3	GGATGGCTCCTGTTGGACTC TGCCAAGACAAGCTCAACCA	186	Reverse transcription-polymerase chain reaction PCR (RT-PCR) of Masou salmon *elovl2* transgene
18s_F 18s_R	GAGAAACGGCTACCACATCC GATACGCTCATTCCGATTACAG	128	Internal control for RT-PCR

2.3.7 Gene quantification

Total RNA was isolated from five tissues: barbel, fin, muscle, liver and kidney using RNeasy Plus Universal Mini Kit (QIAGEN, Valencia, CA), and was reverse transcribed using iScript^{TM} cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. Concentrations of the reverse-transcribed products were adjusted to 250 ng/µL, 1µL was used in 10 µL PCR reaction mixture. The expression level of ribosomal 18s mRNA was used as an internal control (Small et al., 2008). The primers used for Reverse transcription-polymerase chain reaction PCR (RT-PCR) are listed in Table 1. RT-PCR was performed using a DNA Engine Thermal Cycler (Bio-Rad, Hercules, CA). The RT-PCR reaction mixture was denatured at 95 ºC for 3 min and then subjected to 39 cycles of 95 ºC for 30 s, 61 ºC for 15 s and 72 ºC for 15 s, with a final extension time of 10 min at 72 ºC. The PCR products were electrophoresed on a 1% agarose gel.

2.3.8 Fatty acid analysis

Muscle tissues of samples were analyzed for lipids and fatty acid compositions. Lipids were extracted as described by Foch et al. (1957). Fatty acids were transesterified with 14% boron trifluoride (BF_3) (Metcalfe and Schmitz, 1961), and analyzed with Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a capillary column (30m × 0.25mm × 0.25 µm). The oven temperature was 100 ºC, held for 2 minutes, raised to 240 ºC at rate of 4 ºC/min, and held at 240 ºC for 10 minutes. Fatty acids were identified comparing sample

retention times to a standard mix (Supelco® 37 Component FAME Mix). The results of fatty acids were expressed as relative percentage of total identified FAMEs.

2.3.9 Statistical analysis

Fisher's exact test was used to analyze the integration rate with R software (version 4.1.0., R Foundation for Statistical Computing, Vienna, Austria). Statistical analysis of fatty acids composition data was performed by SAS statistical software (version 9.1, SAS Institute, Inc., Cary, NC) using the student's *t*-test. $P < 0.05$ was considered to be statistically significant.

2.4 Results

As shown in Table 2, hatching rates were similar, 9.1% and 9.3% for CRISPR/Cas9 and random integration method, respectively. Integration rate of transgenic fish generated by CRISPR/Cas9 method at seven months was 19.0%, and was not higher than random integration (27.3%) ($P = 0.64$). Mosaicism existed in P1 fish produced by both CRISPR/Cas9 and random integration. Not all five tissues of each transgenic fish had detectable *OmElovl2* gene. PCR results (Figure 3C) showed that the barbel, fin and muscle of transgenic fish #1 produced by CRISPR/Cas9 contained *OmElovl2* gene, but liver and kidney did not. For transgenic fish produced by random integration, only barbel of fish #1, fin and liver of fish #2, barbel and fin of fish #3 carried transgene. In summary,13 of 15 tissues examined carried the transgene in three CRISPR/Cas9 transgenic fish, while *OmElovl2* gene were detected in only five of 15 tissues in three random integration transgenic fish. CRISPR/Cas9 had lower level of mosaicism in P1 transgenic fish than random integration.

Table 2. Results of electroporation and screening of masu salmon, *Oncorhynchus masou*, *elovl2* transgene in putative transgenic channel catfish, *Ictalurus punctatus.*

	Electroporation eggs	Hatching fry	Survived at seven months	Positive founder
CRISPR/Cas9 mediated knock-in	450	41	21	4/21(19.0%)
Random integration	450	42	11	3/11 (27.3%)

OmElovl2 gene was expressed in all three CRISPR/Cas9 electroporated P1 fish (Figure 4). Fish #3 had strong *OmElovl2* gene expression in all five tissues: barbel, fin, muscle, liver and kidney. Fish #1 and #2 had relatively lower expression level than fish #3. There was no detectable transgene expression in liver of fish #1 and barbel of fish #2. For randomly integrated P1 fish, *OmElovl2* expression was not detected in any of five tissues for any fish.

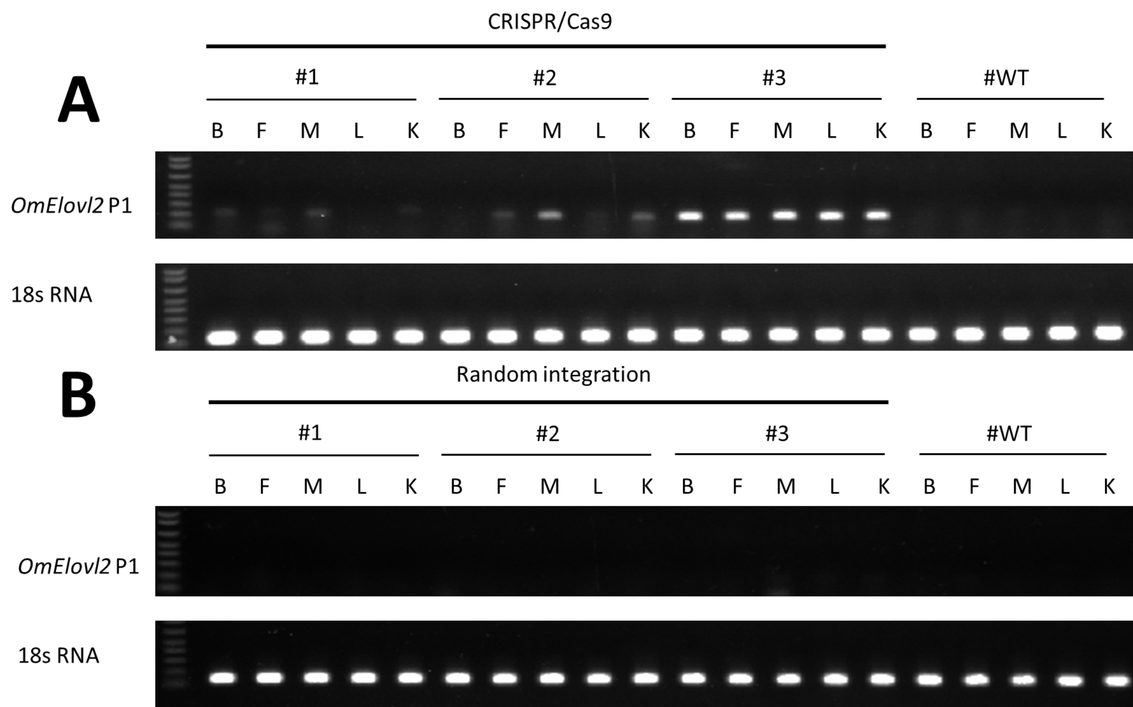

Figure 4. RT-PCR analyses of masu salmon, *Oncorhynchus masou*, *elovl2* gene (*OmElovl2*) expression in different tissues of P1 transgenic channel catfish, *Ictalurus punctatus*.
(A) P1 transgenic channel catfish produced by CRISPR/Cas9 technology.
(B) P1 transgenic channel catfish produced by random integration. The tissues are B: barbel; F: fin; M: muscle; L: liver; K: kidney. WT: wild type control.

Fatty acid composition in muscle of transgenic channel catfish produced by the two methods and corresponding non-transgenic channel catfish controls are showed in Table 3. The 14:0, 18:0 , 20:4 n-3, 22:6 n-3 and 22:5 n-3 fatty acids in transgenic P1 channel catfish produced by CRISPR/Cas9 technology were significantly different from their non-transgenic controls ($P < 0.05$). Fatty acids 14:0, 20:4 n-3 and 22:5 n-3 in CRISPR/Cas9 transgenic fish decreased by 31.0%, 38.1% and 18.5%, respectively, compared to non-transgenic controls. Fatty acids 18:0 and DHA (22:6n-3) levels in CRISPR/Cas9 transgenic fish increased by 10.9 and 20.7% compared with the non-transgenic fish. However, there were no significantly difference for all fatty acid components between transgenic fish produced by random integration and their non-transgenic controls.

Table 3. Fatty acid compositions in muscle of P1 masu salmon, *Oncorhynchus masou*, *elovl2* (*OmElovl2*) transgenic channel catfish, *Ictalurus punctatus* produced by CRISPR/Cas9 technology and random integration, and their full-sibling non-transgenic controls that were grown in aquaria.

Fatty acid (%)		CRISPR/Cas9		Random integration	
Symbol	Common name	Transgenic	Non-transgenic	Transgenic	Non-transgenic
14:0	Myristic acid	2.92 ± 0.06*	4.23 ± 0.37	3.67 ± 0.60	4.06 ± 0.56
16:1 n-7	Palmitoleic acid	6.98 ± 0.09	8.82 ± 0.92	2.73 ± 0.73	2.83 ± 1.43
16:0	Palmitic acid	21.56 ± 0.16	21.09 ± 2.05	33.65 ± 1.58	34.18 ± 5.31
17:0	Heptadecanoic acid	0.22 ± 0.01	0.38 ± 0.12	1.30 ± 0.13	1.22 ± 0.45
18:2 n-6	Linoleic acid (LA)	9.66 ± 0.85	8.08 ± 2.91	5.56 ± 1.43	5.33 ± 2.38
18:1 n-9	Oleic acid	33.31 ± 0.92	32.66 ± 4.60	21.38 ± 1.11	21.76 ± 5.96
18:0	Stearic acid	4.90 ± 0.07*	4.42 ± 0.24	10.37 ± 1.07	9.68 ± 2.13
20:5 n-3	Eicosapentaenoic acid (EPA)	5.18 ± 0.08	5.27 ± 0.28	8.81 ± 1.10	10.12 ± 2.83
20:3 n-6	Eicosatrienoic acid	0.56 ± 0.04	0.68 ± 0.28	2.20 ± 0.34	1.96 ± 0.33
20:4 n-3	Arachidonic acid	0.86 ± 0.06*	1.39 ± 0.16	1.98 ± 0.20	1.89 ± 0.82
20:2 n-6	Eicosadienoic acid	0.67 ± 0.13	0.65 ± 0.29	0.45 ± 0.06	0.49 ± 0.10
20:1 n-9	Eicosenoic acid	1.39 ± 0.07	1.69 ± 0.36	1.05 ± 0.21	0.95 ± 0.48
22:6 n-3	Docosahexaenoic acid (DHA)	9.52 ± 0.24*	7.89 ± 0.49	5.10 ± 0.60	3.98 ± 1.86
22:5 n-3	Docosapentanoic acid (DPA)	2.25 ± 0.04*	2.76 ± 0.11	1.75 ± 0.17	1.57 ± 0.43
Saturates	Saturated fatty acids	29.60 ± 0.17	30.12 ± 2.48	48.99 ± 1.94	49.14 ± 8.42
MUFAs	Monounsaturated fatty acids	41.69 ± 0.99	43.17 ± 4.94	25.16 ± 1.16	25.54 ± 6.99
PUFAs	Polyunsaturated fatty acids	28.71 ± 0.97	26.71 ± 4.47	25.86 ± 1.06	25.32 ± 1.58
n-3	Total omega-3 fatty acids	17.82 ± 0.29	17.31 ± 0.99	17.65 ± 0.75	17.55 ± 1.46
n-6	Total omega-6 fatty acids	10.89 ± 0.80	9.41 ± 3.49	8.21 ± 1.76	7.78 ± 2.61

Saturates: 14:0, 16:0, 17:0, 18:0.

MUFAs: 16:1n-7, 18:1n-9, 20:1 n-9
PUFAs: 18:2n-6, 20:5n-3, 20:3n-6, 20:4n-3, 20:2n-6, 22:6n-3, 22:5n-3
n-3: 20:5n-3, 20:4n-3, 22:6n-3, 22:5n-3
n-6: 18:2n-6, 20:3n-6, 20:2n-6
Values expressed in mean ± SD. "*" indicates statistically significant difference in fatty acid compositions between transgenic and non-transgenic fish (student's *t*-test , $P < 0.05$).

2.5 Discussion

Previously, random integration of gene transfer to fertilized eggs was the only feasible option to produce transgenic fish (Maclean and Laight, 2000; Tonelli et al., 2017) prior to the development of targeted gene transfer with modified endonuclease systems, resulting in homologous recombination. However, the transgenes do not normally integrate into the host genome by this technique during the first division of the eggs, thus mosaicism of the transgenes is universal in the P1 generation. In this study, only five of 15 tissues were detected possessing the transgene for three positive P1 fish produced by random integration. Comparatively, 13 of 15 tissues carried the transgene for three positive P1 fish produced with CRISPR/Cas9. The results indicated that CRISPR/Cas9 reduced mosaicism in the initial generation compared to random integration since CRISPR/Cas9 began functioning prior to first cell division and can continue cleaving the DNA for more than one cell division.

Additionally, the number of copies and the integration sites of the transgene are unpredictable for random integration technique, which might result in alteration of transgene expression with detrimental gene dosage effects and position effects (Lin et al., 1994; Rahman et al., 2000). Our results indicated that no P1 transgenic fish produced by random integration had detectable transgene expression. The lack of detectable transgene expression could be because of the mosaicism. Another explanation could be the silencing of transgenes, which was observed in previous studies due to methylation of transgenes and position effects (Maclean and Laight,

2000; Martin and McGowan, 1995; Rahman et al., 2000). CRISPR/Cas9 technology can target sites precisely with sgRNAs. Good target sites help reduce position effects and obtain high transgene expression.

In this study, a non-coding region in chromosome1 of channel catfish was selected as the target site. RT-PCR results indicated that *OmElovl2* gene was expressed in various tissues of all CRISPR/Cas9 P1 fish. It was reported that the existence of highly conserved non-coding regions (HCNRs) in all vertebrates that were strongly associated with genes, but large regions of the genome, called the gene desert, have no obvious biological role (De La Calle-Mustienes et al., 2005). Thus, targeting a non-coding region, without microRNA loci, long non-coding RNA region or heterochromatin, should reduce the risk of transgene interruption of other functional genes. A previous study also suggested that strong cathelicidin transgene expression was observed in various tissues of P1 fish and had no adverse effect on survival rates by targeting the same non-coding site (Simora et al., 2020).

Fatty acid compositions of fillets are mainly influenced by species (Rahnan et al., 1995) and diets (Hardy et al., 1987), and may also be affected by environment factors (Tocher and Sargent, 1990) or size or age of animals (Kiessling et al., 2001). In this study, transgenic fish produced by CRISPR/Cas9 and random integration methods were compared with their non-transgenic controls, as transgenic fish and their corresponding controls were from same family (full-siblings), cultured in same environment and fed with exactly the same diets. For transgenic P1 fish produced by CRISPR/Cas9, fatty acids 14:0, 20:4 n-3 and 22:5 n-3 decreased by 31.0%, 38.1% and 18.5%, respectively, fatty acids 18:0 and DHA (22:6n-3) content increased by 10.9 and 20.7%. *Elovl2* gene had great activity in the elongation of C20 and 22 PUFA substrates (Morais et al., 2009; Sun et al., 2020), thus fish are dependent on *elovl2* for DPA to 24:5n-3

synthesis and ultimately DHA synthesis (Gregory and James, 2014; Liu et al., 2020). Knocking out the *elovl2* gene reduced the content of DHA in Atlantic salmon (Datsomor et al., 2019) and zebrafish (Liu et al., 2020). Thus, it is logical that knocking in the *OmElovl2* transgene could reduce C20 and C22 PUFA substrates and improve DHA levels in transgenic channel catfish in our study.

Fatty acid changes for randomly integrated transgenic P1 fish were somewhat similar to those for the P1 produced via CRISPR/Cas9. The fatty acid 14:0 also decreased, but less so 9.6%. Both 20:4 n-3 and 22:5 n-3 increased, 5.8% and 11.5%, respectively, in contrast to the 20-40% for the CRISPR/Cas9 generated fish. However, the observed 18:0 and DHA (22:6n-3) end product level increases of 7.1 and 28.1% were quite similar and actually numerically higher for DHA for the randomly integrated P1, although none of these changes were significantly different for the randomly generated P1. The reason for the lack of significance was that the variability in the means was much higher in these fish, perhaps because of the much greater mosaicism of these fish.

However, since the two sets of fish, randomly integrated and target generated were made with different sets of families, this increased variability may have been a family effect as the controls for the randomly integrated set had considerably higher standard deviations/coefficients of variation compared to the controls of the CRISPR/Cas9 generated fish. Of course, both mosaicism and family effects could have contributed to the variation or lack thereof in the transgenic channel catfish. This natural variation likely effects statistical analysis in many studies of n-3 fatty acids in channel catfish. The overall mean for n-3 fatty acids was surprisingly similar in both sets of families and between transgenic individuals and controls, however, when examining distributions of the other forms of fatty acids, there was a myriad of differences.

Results with *OmEvol2* transgenic nibe croaker were different than our results with channel catfish. Transgenic nibe croaker produced by random integration showed expression of *OmElovl2* transgene in several tissues, but did not produce higher content of DHA than non-transgenic fish (Kabeya et al., 2014). Again, this might be explained by mosaicism. It is not uncommon for the same transgene to have different effects when introduced to different species (Dunham, 2010). However and again, these comparisons are difficult because of position effects.

2.6 Conclusion

The present study produced P1 transgenic channel catfish carrying the *OmElovl2* transgene by CRISPR/Cas9 technology and random integration. P1 transgenic fish produced by CRISPR/Cas9 technology showed a lower level of mosaicism, higher transgene expression, and better effects on DHA improvement compared to transgenic fish produced by random integration. The results suggest that CRISPR/Cas9 technology would help accelerate transgenic breeding procedures and reduce breeding cost and risk.

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Chapter 3 CRISPR/Cas9-mediated transgenesis of the masu salmon (*Oncorhynchus masou*) *elovl2* gene improves n-3 fatty acid content in channel catfish (*Ictalurus punctatus)*

3.1 Abstract

Omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), play a very important role in human health. Channel catfish (*Ictalurus punctatus*), is one of leading freshwater aquaculture species in the US, but has low levels of EPA and DHA compared to some fish such as salmon. To improve EPA and DHA content, a modification of the n-3 PUFA biosynthetic pathway was achieved through the insertion of an *elovl2* transgene isolated from masu salmon (*Oncorhynchus masou*) driven by a carp β-actin promoter using a two-hit by gRNA and two oligos with a targeting plasmid (2H2OP) CRISPR/Cas9 approach. Integration rate of the transgene was high (37.5%) and detected in twelve different tissues of P_1 transgenic fish with tissue-specific gene expression. Liver and muscle had relative high gene expression (13.4 and 9.2 fold-change, respectively). Fatty acid analysis showed DHA content in the muscle from transgenic fish was 1.62 fold higher than in non-transgenic fish ($P < 0.05$). Additionally, total n-3 PUFAs and omega-6 polyunsaturated fatty acids (n-6 PUFAs) increased to 1.41 fold and 1.50 fold, respectively, suggesting the β-actin-*elovl2* transgene improved biosynthesis of PUFAs in channel catfish as a whole. The n-9 fatty acid level decreased in the transgenic fish compared to the control. Morphometric analysis showed that there were significant differences between injected fish with sgRNAs (including positive and negative fish) and sham injected controls ($P < 0.001$). Potential off-target effects are likely the major factor responsible for morphological deformities. Optimization of sgRNA design to maximize activity and reduce off-target effects of

CRISPR/Cas9 should be examined in future transgenic research, but this research shows a promising first step in the improvement of n-3 PUFAs in channel catfish.

3.2 Introduction

Long-chain polyunsaturated fatty acids (n-3-PUFAs), principally eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are widely recognized to have benefits for human health (Belkind-Gerson et al. 2008; Innis 2008; Janssen and Kiliaan 2014; Nestel et al. 2015). The channel catfish, *Ictalurus punctatus*, is the most important freshwater aquaculture species in the USA, but unlike marine fish it has low levels of n-3 PUFAs (Robinson et al. 2001). *Elovl2* is an essential elongase gene in the DHA biosynthetic pathway (Gregory and James 2014). A previous study showed that transgenic nibe croakers (*Nibea mitsukurii*) carrying an *elovl2* gene isolated from masu salmon, *Oncorhynchus masou*, have 2.28-fold higher DPA (docosapentaenoic acid) than non-transgenic fish (Kabeya et al. 2014). DPA is an intermediate in the synthesis of DHA. DPA can be converted to DHA by two further elongations, a second Δ6 desaturation and a peroxisomal chain shortening step, or by only one step of Δ4 desaturation (Li et al. 2010). Overexpression of elongase-like gene from masu salmon in transgenic zebrafish helped improved their DHA by 1.33-fold, compared with non-transgenic fish (Alimuddin et al. 2008). Therefore, production of transgenic channel catfish carrying *elovl2* transgene might increase their DHA content, thus making channel catfish an even healthier food for humans.

Two-hit by gRNA and two oligos with a targeting plasmid (2H2OP) is a highly efficient knock-in method, which uses two guide RNAs (gRNAs) to cut target sites in genomic DNA and the donor plasmid DNA, and uses two short ssODNs (single-stranded oligo DNA nucleotides) to ligate the ends of cut sites (Yoshimi et al. 2016). Gene editing by knocking out luteinizing hormone (LH) can achieve sterilization (Qin et al. 2016) to reduce potential deleterious

environmental risks of transgenic fish. Simultaneous gene knockout and knockin would allow more rapid attainment of these goals. Although the targeting specificity of Cas9 is believed to be tightly controlled by the 20-nt guide sequence of the sgRNA and the presence of a PAM adjacent to the target sequence in the genome, potential off-target cleavage activity could still occur in DNA sequences with even three to five base pair mismatches in the PAM-distal part of the sgRNA-guiding sequence (Zhang et al. 2015). Thus, high frequency of off-target activity (≥ 50%) could be induced by RNA-guide endonuclease at unintended sites other than the intended on-target site, which may disrupt the functionality of other normal genes, and may also affect the phenotype (Fu et al. 2013; Zhang et al. 2015).

Geometric morphometrics is a popular statistical analysis of form based on landmark coordinates (Armbruster 2012; Takács et al. 2016). After separating shapes from overall size, position and orientation of the landmark configurations, Procrustes shape coordinates are produced for statistical analysis, allowing comparison of shape differences or visualization of shape deformations (Mitteroecker and Gunz 2009). Geometric morphometrics method had been applied broadly in differentiating species (Doadrio et al. 2002; Ponton 2006) and also describing intraspecific differences (Jørgensen et al. 2008; Herler et al. 2010). Additionally, geometric morphometrics can also detect growth pattern differences of transgenic fish, compared to the wild type.

In this study, our overall long-term goal was to use the 2H2OP method to knock in *elovl2* gene isolated from masu salmon targeting at *LH* exon2 site of genome in channel catfish to make *LH* gene lose its function while increasing 3-n fatty acid levels. Specific objectives were to produce transgnic lines of channel catfish carrying masu salmon *elovl2* gene with a high level of

EPA and DHA contents, and detect and analyze the reasons of the alteration of morphometrics in transgenic fish.

3.3 Methods

3.3.1 Ethical statement

Channel catfish were reared at the Fish Genetics Research Unit, E.W. Shell Fisheries Center, School of Fisheries, Aquaculture and Aquatic Sciences at Auburn University, Alabama, USA. The Institutional Animal Care and Use Committee (IACUC) approved all the experiments in this study.

3.3.2 Construction of plasmids

FRM2bl_Δ5_desaturase plasmid with carp β-actin promoter (Cheng et al. 2014) (NCBI accession#: AF170915.1) was used as a backbone construct and then modified. To replace the sequence for the *O. masou delta5*-desaturase like gene, the plasmid was linearized by KpnI and EcoRI enzyme digestion and then was excised form the backbone and replaced with Masu salmon *elovl2* sequence which was provided by Goro Yoshizaki, Tokyo University of Marine Science and Technology (Kabeya et al. 2014).

3.3.3 Design and preparation of sgRNA and CRISPR/Cas9 system

Software *sgRNAcas9_3.0.5* (Xie et al., 2014) was used to design sgRNAs targeting at plasmid and LH site (Figure 5A). The sgRNAs and ssODNs are shown in Table 4. The cloning free (PCR-based) method with two oligos was used to generate sgRNA templates (Varshney et al. 2015), one oligo containing the T7 promoter and the 20-nt gene-specific target sequence, the other one, the universal primer containing the sgRNA scaffold. The sgRNA templates were generated by T7 run-off as described previously (Gagnon et al. 2014; Varshney et al. 2015) with modifications (Khalil et al. 2017). EconoTaq® Plus 2x Master Mix (Lucigen, Middleton, WI)

was used to anneal the two oligos. The sgRNA template was used to transcribe RNA by Maxiscript T7 Kit (Thermo Fisher Scientific, Waltham, MA). The sgRNAs generated were purified using Zymo RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). The Cas9 protein was obtained from PNA BIO Inc. (Newbury Park, CA).

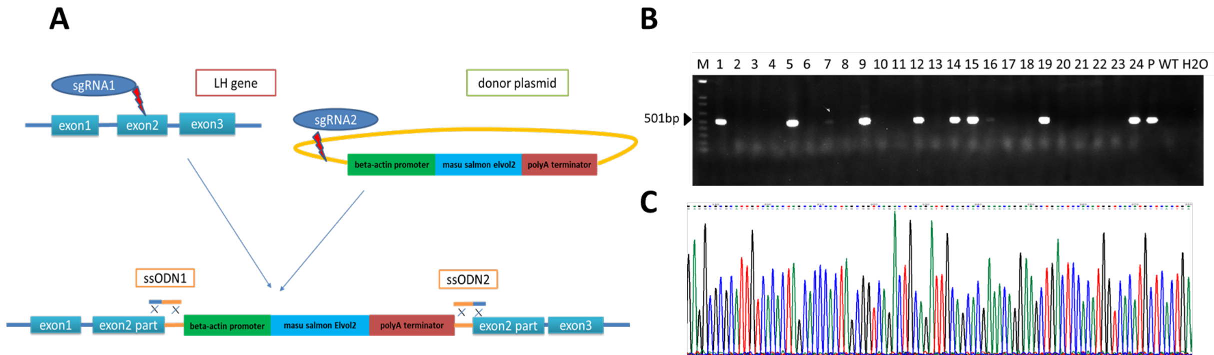

Figure 5. Strategy and molecular characterization of gene transfer of the common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgene integration into channel catfish, *Ictalurus punctatus*
(A) Schematic representation of two-hit by gRNA and two oligos with a targeting plasmid (2H2OP).
(B) PCR analysis of masu salmon *elovl2* transgene integration into channel catfish. M: marker; P: positive control; WT: wild type control; H2O: water negative control.
(C) PCR product sequencing result.

Table 4. Primers used for CRISPR/Cas9 knockin and mutation screening for masu salmon, *Oncorhynchus masou, elovl2* transgene and the luteinizing hormone (*LH)* gene target site in putative transgenic channel catfish, *Ictalurus punctatus*

Sequence	Primer name	Description
TTCAAACCGCCATCTGCAGC	sgRNA1	For sgRNAs synthesis
CTTGTCTGTAAGCGGATGCC	sgRNA2	
AAGTAAAATATATATTCACCTTGGTGAAGCAGTG CCCGCTGCCGGGAGCAGACAAGCCCGTCAGGGCG CGTCAGCGGGTG	ssODN1	For assistance of ligating the ends of cut sites
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAA GCGGATGCAGATGGCGGTTTGAAACACAAGGCAT TTCGGGCAGCCA	ssODN2	
CGAAATCCGTTCCTTTTTACTG	elovl2_F1	Masu salmon *elovl2* transgene detection
CTGGCCTGTTCCTCATGTATTT	elovl2_R1	
GGATGCCTGCTCTTCCAGTT	elovl2_RT_F1	For quantitative real-time PCR of masu salmon *elovl2* transgene
GTGCCATTGGTGGAGACAGA	elovl2_RT_R1	
GAGAAACGGCTACCACATCC	18s_F	Internal control for quantitative real-time

GATACGCTCATTCCGATTACAG	18s_R	PCR
TCGATGGTACAGTCCGAGGT	LH_elovl2_F1	Upstream junction detection
CATAGTTAAGCCAGCCCCGA	LH_elovl2_R1	
GGTTTCATAGCGAACGTCCC	LH_elovl2_F2	
TACAATCTGCTCTGATGCCGC	LH_elovl2_R2	
TCATTCTTGGAGTAAATGGACTCGT	LH_elovl2_F5	
TCATCAGCAGCACAGAAGACT	LH_elovl2_R5	
GGCAGGCGTACGGTTTCATA	LH_elovl2_C_F7	
ATGTTAGACGAGACTACGGCG	LH_elovl2_C_R7	
GATGGTACAGTCCGAGGTGTC	LH_elovl2_C_F8	
TTTGGGATGTGGTTGTCGGA	LH_elovl2_C_R8	
TGAGATCCAGTTCGATGTAACCC	elovl2_LH_F3	Downstream junction detection
GTCAGTGCCAGCTTCCTCTTT	elovl2_LH_R3	
CTCAAGGATCTTACCGCTGTTG	elovl2_LH_F4	
CCCCGCTCAAAGCTACATTCT	elovl2_LH_R4	
AATAGTGTATGCGGCGACCGA	elovl2_LH_F6	
CACGTTTCGATTCTGCGACA	elovl2_LH_R6	
CAGGAGGCTAGCAACAGTCTTC	elovl2_LH_C_F9	
CCGCTCAAAGCTACATTCTGC	elovl2_LH_C_R9	

3.3.4 Transgenic fish production and rearing

Brood stock spawning followed the procedures of Qin et al. (2016). Microinjection was performed as described by Khalil et al. (2017) with modification. A combination of 200ng/µL Cas9 protein, 80ng/µL sgRNA, 12.5ng/µl donor plasmid, 12.5ng/µl short ssODNs and 12% phenol red were co-injected into one-cell stage channel catfish embryos. Control embryos were only injected with 12% phenol red. The microinjection volume of the solution was 5 nL for each treatment embryo and for each sham-injected control embryo. Then all microinjected embryos were reared in Holtfreter's solution (Bart and Dunham 1996) containing 10 ppm doxycycline at 27 °C until hatching. Fry were transferred into a recirculating system.

Injected fish for transgene insertion and for control were separated into different 60 L tanks with same density (2 fry/L). Fry were first fed Aquamax fry powder (50% crude protein, 17% crude fat, 3% crude fiber and 12% ash) (Purina Animal Nutrition LLC, Shoreview, MN)

four times a day for two months. Fingerlings were then fed Aquaxcel WW Fish Starter 4512 (45% crude protein, 12% crude fat, 3% crude fiber and 1% phosphorus) (Cargill Animal Nutrition, Minneapolis, MN) twice a day for four months. When six-months-old, injected fish (weighing 30-50g) for transgene insertion and controls were pit tagged and fin clipped, and then mixed together into 500L tanks and the density adjusted to one fish per 10 L. Fin samples were stored at -80 °C for DNA extraction. Juvenile fish were fed with WW 4010 Transition (40% crude protein, 10% crude fat, 4% crude fiber and 1% phosphorus) (Cargill Animal Nutrition, Minneapolis, MN) once a day to satiation.

3.3.5 Integration rate

DNA was extracted as previously described (Kurita et al. 2004). Transgenic fish samples were screened by PCR with a specific forward primer: elovl2_F1 and reverse primer: elovl2_R1 (Table 4). Primers were designed using the NCBI primer design. The PCR amplification procedure was as follows: initial denaturation for 3 min at 95ºC, followed by 36 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s and 40 s extension at 72ºC, and final elongation at 72ºC for 5 min. The PCR products were analyzed by electrophoresis with 1 % agarose gels. PCR products were verified by sequencing (Genewiz, South Plainfield, NJ) to identify the fish that were positive for gene insertion.

3.3.6 Mosaicism and gene expression analysis of the transgene

Three 10-month-old positive fish and three sham injected control fish were randomly selected and sacrificed. Twelve tissues, barbel, fin, muscle, skin, liver, heart, kidney, spleen, intestine, brain, swim bladder and gill, of each fish were sampled followed by extraction of DNA and RNA. Normal PCR and qRT-PCR were run to detect mosaicism and expression of the transgene. Total RNA was isolated from various tissues using RNeasy Plus Universal Mini Kit

(QIAGEN, Valencia, CA), and was reverse transcribed using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed on C1000 Thermal Cycler (Bio-Rad, Hercules, CA) using the of SsoFast^{TM} EvaGreen® Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The expression level of ribosomal 18s mRNA (Small et al., 2008) was used as an internal control. The forward primer (elovl2_RT_F1) and reverse primer (elovl2_RT_R1) used for qRT-PCR are listed in Table 1. The reaction conditions were as follows: 94ºC for 5 s, followed by 40 cycles of 94ºC for 5 s, 60ºC for 5 s, and a dissociation curve profile of 65-95ºC for 5 s/0.5ºC increment. Results were expressed relative to the expression levels of 18S rRNA in each sample using the CFX Manager Software version 1.6 (Bio-Rad, Hercules, CA), and crossing-point (CT) values were converted to fold differences using the relative quantification method (Schmittgen and Livak 2008).

3.3.7 Off -target effect analysis

Specific primers were designed by NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/ tools/primer-blast/) to test junction parts between genome and plasmid. Online tool Cas-OFFinder (http://www.rgenome.net/cas-offinder/) (Bae et al. 2014) was used to predict off-target sites, but actual off-target analysis was not conducted because the predicted sites were too numerous.

3.3.8 Fatty acid analysis

Total lipids were extracted from muscle of three transgenic and non-transgenic fish each following the protocol from Folch et al. (1957). Fatty acid methyl esters (FAME) were prepared by transesterification from lipids using the standard boron tri-fluoride-methanol method (Morrison and Smith 1964) and analyzed on Agilent 6890N gas chromatograph (Agilent

Technologies, Santa Clara, CA) equipped with a capillary column (30m × 0.25mm × 0.25 µm). A 1 µL sample was injected by split injection. Split ratio was 30:1. The oven temperature was 100 ºC, held for 2 minutes, raised to 240 ºC at rate of 4 ºC/min, and held at 240 ºC for 10 minutes. Fatty acids were identified comparing sample retention times to a standard mix (Supelco® 37 Component FAME Mix). The results of fatty acids were expressed as relative percentage of total identified FAMEs.

3.3.9 Morphometric analysis

Sixteen-month-old fish were placed on the same plane and pictures were taken in lateral views. Pictures of 19 positive fish, 28 negative fish and 13 sham injected control fish were digitized with 13 landmarks using StereoMorph R package (Olsen and Westneat 2015) (Figure 7A). Then a generalized procrustes analysis (GPA) was performed to produce plots of specimens for each group (Figure 7B). Procrustes ANOVA with permutation procedures were used to assess statistical hypotheses describing patterns of shape variation and covariation for Procrustes-aligned coordinates among groups. Pairwise comparisons, discriminant analysis and deformation grid were also analyzed using R packages: geomorph (Adams and Otárola-Castillo 2013) and Morpho (Schlager 2017).

3.3.10 Statistical analysis

Gene expression and fatty acid composition between transgenic and non-transgenic fish were analyzed with a Student's two-sample *t*-test ($P < 0.05$) using SAS statistical software (version 9.1, SAS Institute, Inc., Cary, NC). Procrustes ANOVA with permutation procedures were used to assess shape differences of three groups and pairwise comparison was corrected by Bonferroni correction ($P < 0.05/3$; thus, 0.017 is the accepted level of significance).

3.4 Results

3.4.1 Integration rate

PCR (Figure 5B) and sequencing results (Figure 5C) indicated that the integration rate was 37.5% (81/216).

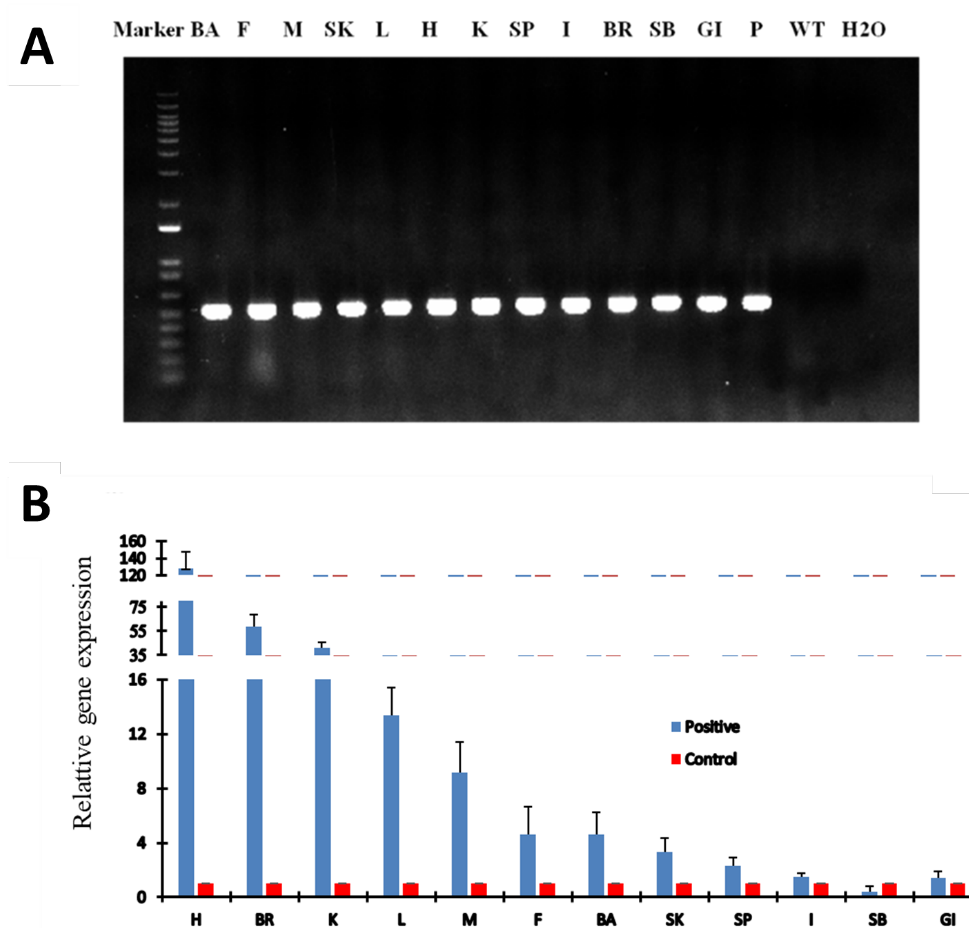

Figure 6. Gene transfer and expression of the common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgene in channel catfish, *Ictalurus punctatus* grown in aquaria
(A) PCR analysis of different tissues.
(B) Relative gene expression of different tissues. BA: barbel; F: fin; M: muscle; SK: skin; L: liver; H: heart; K: kidney; SP: spleen; I: intestine; BR: brain; SB: swim bladder; GI: gill; P: positive control; WT: wild type control; H2O: water negative control; Control is baseline and does not express the transgene, thus expression is zero. It appears positive due to transformation for statistical analysis (Student's two-sample *t*-test; $P < 0.05$).

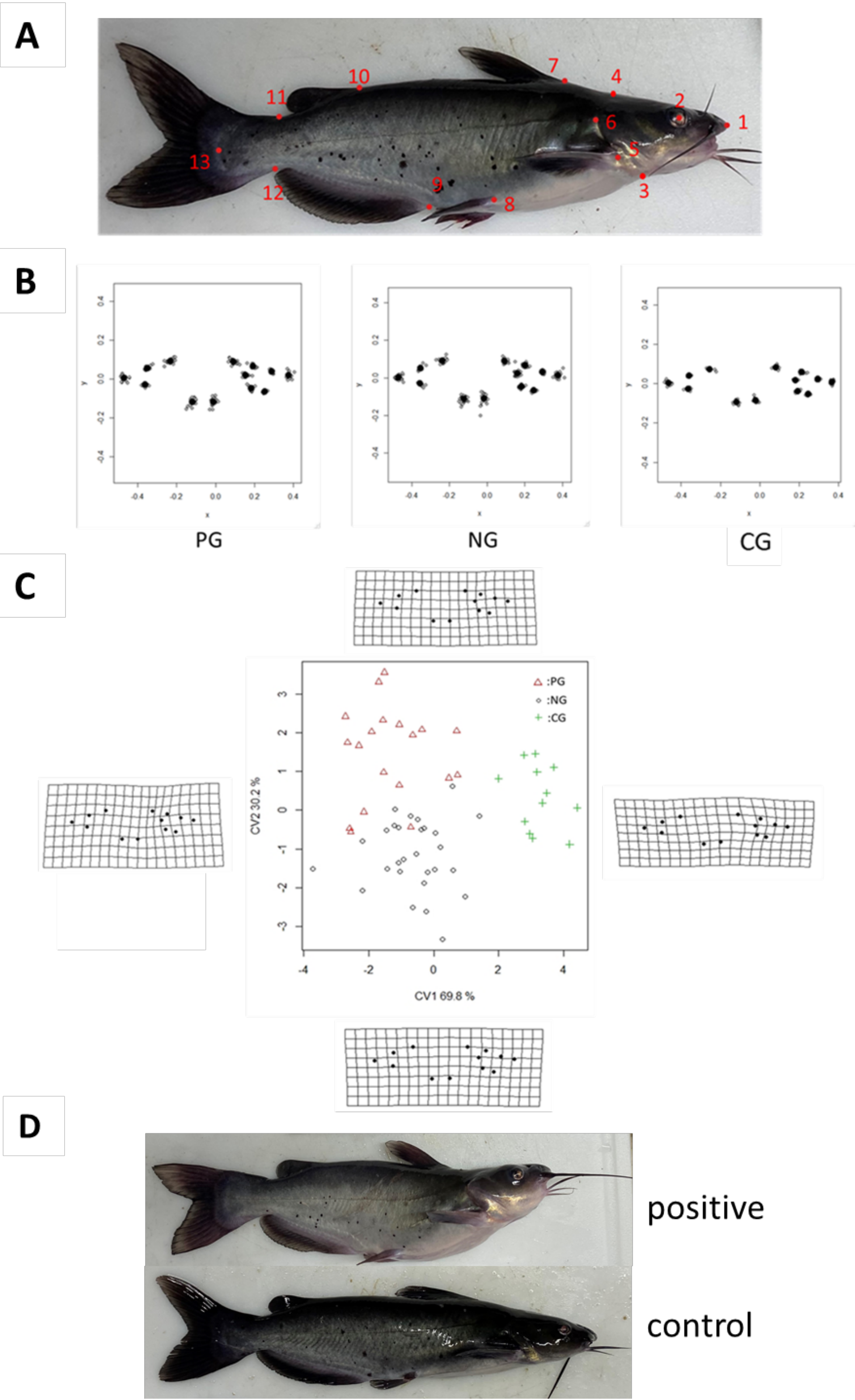

Figure 7. Morphology of transgenic common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* and control channel catfish, *Ictalurus punctatus*.

(A) Lateral view with 13 landmarks: 1 Snout tip; 2 Center of the eye; 3 Intersection of gill opening and ventral margin of body; 4 Supraoccipital, posteromedial tip; 5 Origin of pectoral fin; 6 Posteriormost edge of opercle; 7 Origin of dorsal fin; 8 Origin of pelvic fin; 9 Origin of anal fin; 10 Origin of adipose fin; 11 Origin of anterior dorsal procurrent caudal-fin ray; 12 Origin of anterior ventral procurrent caudal-fin ray; 13 End of vertebral column.
(B) Scatter plot of landmark configurations after Procrustes superimposition. PG: positive group, NG: negative group, CG: sham-injected control group.
(C) Canonic variate plot and deformation grid. PG: positive group, NG: negative group, CG: sham-injected control group. CV1: the first canonical variable, CV2: the second canonical variable. d Body shape of positive fish (top) and control fish (bottom)

3.4.2 Mosaicism and gene expression

All 12 tissues including barbel, fin, muscle, liver, heart, skin, kidney, spleen, intestine, brain, swim bladder and gill carried the masu salmon *elovl2* transgene in the three positive fish (Figure 6A). RT-PCR results (Figure 6B) showed all tissues, except swim bladder and gill of the positive fish, had significantly greater changes ($P < 0.05$). High variation was observed among different tissues. Heart, brain and kidney had the most abundant transgene mRNA (41.5-128.4 fold). Liver and muscle had 13.4 and 9.2 fold-change, respectively. Intestine and spleen showed lower changes (1.47-2.33 fold). Masu salmon *elovl2* transgene did not express in the swim bladder and gill.

3.4.3 Off-target effect

Nine pairs of primers did not amplify junction regions of plasmids and target sites. And 458 potential off-target sites of two sgRNAs were predicted (Table 5), which indicates exogenous gene might knocked in unintended sites in genome of channel catfish with high off-target effects or mutations could occur without gene insertion at the off-target sites.

Table 5 Potential off-targets by prediction using online tool Cas-OFFinder (http://www.rgenome.net/cas-offinder/) (Bae et al., 2014) when using CRISPR/Cas9 to knockin the common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgene for channel catfish, *Ictalurus punctatus* with luteinizing hormone gene as the target site

sgRNA	Target sequence	Mismatch bases	Number of off-targets predicted

sgRNA1	TTCAAACCGCCATCTGCAGCNGG	3	3
sgRNA1	TTCAAACCGCCATCTGCAGCNGG	4	27
sgRNA1	TTCAAACCGCCATCTGCAGCNGG	5	260
sgRNA2	CTTGTCTGTAAGCGGATGCCNGG	4	15
sgRNA2	CTTGTCTGTAAGCGGATGCCNGG	5	153

3.4.4 Fatty acid analysis

Fatty acid profiles of transgenic and non-transgenic fish were presented in Table 6. The content of DHA in transgenic fish was 1.62 fold higher than non-transgenic fish. In addition, masu salmon *elovl2* in transgenic fish increased 18:2n-6 by 1.51 fold, 20:2n-6 by 1.46 fold, 22:5n-3 (DPA) by 1.35 fold, total PUFAs by 1.44 fold, total omega-3 polyunsaturated fatty acids (n-3 PUFAs) by 1.41 fold, and omega-6 polyunsaturated fatty acids (n-6 PUFAs) by 1.50 fold, compared with non-transgenic fish ($P < 0.05$). Transgenic fish had lower 18:1n-9, total saturates and total monounsaturated fatty acids (MUFAs) than non-transgenic fish ($P < 0.05$). There was no significant difference for EPA between transgenic and non-transgenic fish ($P > 0.05$).

Table 6 Fatty acid compositions (% of total fatty acids) of muscle from P_1 common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgenic channel catfish, *Ictalurus punctatus* and their full-sibling non-transgenic controls that were grown in aquaria

Fatty acid (%)		Transgenic	Non-transgenic
Symbol	Common name		
14:0	Myristic acid	3.42 ± 0.78	3.41 ± 0.14
15:0	Pentadecanoic acid	0.27 ± 0.06	0.24 ± 0.04
16:2n-4	Hexadecadienoic acid	0.40 ± 0.13	0.30 ± 0.10
16:1n-7	Palmitoleic acid	6.66 ± 1.24	7.50 ± 0.49
16:0	Palmitic acid	18.52 ± 1.91	21.30 ± 0.57
17:1n-7	Heptadecenoic acid	0.26 ± 0.07	0.14 ± 0.08
17:0	Heptadecanoic acid	0.25 ± 0.05	0.18 ± 0.06
18:2n-6	Linoleic acid (LA)	13.48 ± 1.70*	8.93 ± 2.07
18:1n-9	Oleic acid	29.45 ± 0.92*	36.40 ± 3.26
18:0	Stearic acid	4.22 ± 0.92	4.91 ± 0.37
20:5n-3	Eicosapentaenoic acid (EPA)	5.09 ± 0.43	4.32 ± 0.32
20:3n-6	Eicosatrienoic acid	0.73 ± 0.08	0.54 ± 0.14
20:4n-3	Arachidonic acid	0.99 ± 0.29	1.02 ± 0.09
20:2n-6	Eicosadienoic acid	0.69 ± 0.02*	0.47 ± 0.09
20:1n-9	Eicosenoic acid	1.40 ± 0.31	1.32 ± 0.10
21:5n-3	Heneicosapentaenoate acid	0.36 ± 0.09	0.16 ± 0.14
22:6n-3	Docosahexaenoic acid (DHA)	9.86 ± 0.32*	6.09 ± 1.39
22:5n-3	Docosapentanoic acid (DPA)	2.80 ± 0.36*	2.08 ± 0.16
24:6n-3	Nisinic acid	0.64 ± 0.11	0.39 ± 0.18

24:5n-3	Tetracosapentaenoic acid	0.48 ± 0.12	0.29 ± 0.18
Saturates	Saturated fatty acids	26.69 ± 1.52*	30.04 ± 1.12
MUFAs	Monounsaturated fatty acids	37.77 ± 1.89*	45.36 ± 3.19
PUFAs	Polyunsaturated fatty acids	35.54 ± 1.57*	24.60 ± 3.61
Total n-3	Total omega-3 fatty acids	20.23 ± 1.05*	14.36 ± 1.55
Total n-6	Total omega-6 fatty acids	14.91 ± 1.76*	9.94 ± 2.27

Saturates: 14:0, 15:0, 16:0, 17:0, 18:0
MUFAs: 16:1n-7, 17:1n-7, 18:1n-9, 20:1n-9
PUFAs: 16:2n-4, 18:2n-6, 20:5n-3, 20:3n-6, 20:4n-3, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3
Total n-3: 20:5n-3, 20:4n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3
Total n-6: 18:2n-6, 20:3n-6, 20:2n-6
* Significant difference in fatty acid compositions between transgenic fish and non-transgenic fish (Student *t* test; $p < 0.05$)

3.4.5 Morphology

Permutation tests indicated that mean body shapes of injected fish were significantly influenced by both centroid size ($P < 0.004$ for 1000 permutations) and groups ($P < 0.001$ for 1000 permutations). Pairwise comparisons showed there were significant differences between the shapes of the positive transgenic group and sham injected control group ($P < 0.001$), negative transgene injected group and the sham injected control group ($P < 0.001$). The mean shapes of the positive group and the negative group were different ($P = 0.098$). Cross-validated classification showed 90% accuracy. Three negative fish were misclassified into positive group. Three positive fish were misclassified into the negative group. The first discriminant function explained 69.8% and the second function explained 30.2% of variation in shape. Means of shapes in the discriminant space were significantly different ($P < 0.05$) between the control group and other groups. The deformation grid (Figure 3C) suggests that most deformation associated with first canonical variable (CV1) was located along the measured edges of the operculum and dorsal fin (Figure 3D).

3.5 Discussion

A relatively high overall integration rate, 37.5%, was observed for the transfer of the common carp β-actin-masu salmon, *Oncorhynchus masou, elovl2* transgene into channel catfish using 2H2OP. Mosaicism was common, but reduced, in CRISPR/Cas9-mediated gene editing (Mehravar et al. 2019) because the CRISPR/Cas9 system can continuously target and cleave genes at different cell stages (Mizuno et al. 2014; Oliver et al. 2015). In our study, the foreign gene was tested in 12 tissues, including barbel, fin, muscle, liver, heart, skin, kidney, spleen, intestine, brain, swim bladder and gill with the transgene detected in all 12 tissues. The 2H2OP CRISPR/Cas9 method reduced mosaicism efficiently in P1 channel catfish compared to non-targeted traditional approaches (Cheng et al. 2014). However, different tissues showed different levels of gene expression of the foreign gene. Heart, brain and kidney had higher gene expression (41.5-128.4 fold). Muscle, an important edible tissue for fat deposition, had a 9.2-fold change. Intestine and spleen showed lower fold changes (1.47-2.33 fold). The transgene did not have gene expression in the swim bladder and gills. Tissue-specific gene expression was also observed in previous transgenesis studies (Kabeya et al. 2014; Zhang et al. 2019). Liver is the most important tissue for n-3 PUFA synthesis (Pearce 1983; Rapoport et al. 2007). Liver had relatively high gene expression (13.4 fold higher than the control, which was standardized to a base level of one) in this study, which represented the tissue with the 4th highest expression. The transgene plasmid design resulted in successful modification of the fatty acid metabolic pathway and increased n-3 fatty acid levels by 35-62%.

Elovl2 gene has been demonstrated to encode one of the key enzymes for the *in vivo* synthesis of PUFA from their precursors and has the greatest activity for the elongation C20 and C22. *Elovl2* is an especially critical enzyme in the formation of DHA (Leonard et al. 2002;

Gregory and James 2014; Pauter et al. 2017; Pan et al. 2020). *Elovl2*-KO salmon, zebrafish and *elovl2* deficient mice showed reduced levels of DHA and accumulation of EPA (Pauter et al. 2014; Datsomor et al. 2019; Liu et al. 2020). In contrast, over-expression of *elovl2* gene by the transgenic marine fish nibe croaker decreased the level of EPA, but increased the level of DPA (Kabeya et al. 2014). While, over-expression of masu salmon elongase-like gene in transgenic zebrafish improved EPA content by 1.3 fold and DHA by 1.33 fold (Alimuddin et al. 2008), DHA content in the muscle of transgenic channel catfish carrying masu salmon *elovl2* gene was 1.62 fold higher in the current study than in non-transgenic full-siblings. However, the observed mean of EPA was 18.2% larger for transgenic channel catfish in the current experiment compared to full-sibling non-transgenic controls, though not significantly different. Over-expression of *elovl2* enhanced the ability to synthesize C22 in transgenic channel catfish. The effects that *elovl2* transgene has on the phenotype might vary depending upon the host species (Cheng et al. 2010) and culture environment (Zeller et al. 2010). Additionally, transgenic channel catfish showed increased ALA, 20:2n-6, DPA, total PUFAs, n-3 PUFAs, n-6 PUFAs and decreased total saturates, 18:1n-9, total MUFAs, which indicated that *elovl2* is not only a critical enzyme in biosynthesis of DHA, but also a rate limiter for the entire PUFA pathway. The *elovl2* transgene from masu salmon with assistance from other elongase genes (Alimuddin et al. 2008), might improve the elongation of C_{18}-C_{22} PUFA substrates, and up-regulate other desaturase genes (Kjær et al. 2016), increasing the PUFA compositions in channel catfish.

Off-target mutation frequency can be high when using CRISPR/Cas9 technology (Pattanayak et al. 2013) because Cas9 with sgRNA has potential off-target cleavage activity on DNA sequences even with three to five base pair mismatches in the PAM-distal part of sgRNA sequence (Fu et al. 2013). Off-target mutations may cause genomic instability and interrupt other

normal genes functions (Zhang et al. 2015) , which may disrupt normal physiological activity and change the phenotype. In this study, both transgenic positive siblings and negative siblings were co-injected with two sgRNAs, which might increase the chance of off-target effects. Intended target site integrations were not detected with nine paired specific primers. Off-targets predictions showed 458 potential targeted sites. This indicates the potential for high frequency of off-target mutations when two sgRNAs are co-injected in fish. Next-generation sequencing methods like ChIP-seq need to be applied to identify off-target sites in the next research phase (Zhang et al. 2015).

Fish morphometrics are sensitive phenotype traits that reflect changes of normal development and physiology. Previous studies showed morphological deformities of cultured fish were caused by many factors including genetic, environmental, nutritional, disease and toxic factors (Kincaid 1976; Mehrle et al. 1982; Divanach et al. 1997; Baeverfjord et al. 1998; Cheng et al. 2000; Madsen et al. 2001). In this study, body shape differences or visualization of shape deformations among transgenic full-siblings, negative full-siblings and control group were determined using geometric morphometrics based on landmarks. Mean body shapes of fish were significantly different among groups. There were differences between the shapes of positive group and control group, negative group and control group based upon pairwise comparisons, while the mean shapes of positive group and negative group were not different. Since the potential for high off-target frequency existed in this study, sgRNAs targeted sites and transgene insertion sites might disrupt important genes related to morphological deformities (Ostenfeld et al. 1998; Liang et al. 2019; Palasin et al. 2019). Since both of the groups injected with sgRNAs exhibited abnormal morphology, these were likely due to mutations without gene insertions. Damage from microinjection is likely not an explanation as sham injected controls had normal

appearance. Therefore, optimizing sgRNA design to maximize activity and reduce off-target effects of CRISPR/Cas9 is a crucial strategy in transgenic research (Doench et al. 2016). New methodologies for detecting off-target mutations efficiently are needed, enabling better interpretation of genotype-phenotype correlations (Zhang et al. 2015). However, the expectation is that adverse off-target effects would be selected against.

In conclusion, we successfully generated P_1 transgenic channel catfish carrying masu salmon *elovl2* gene with high integration rates using 2H2OP CRISPR/Cas9 technology. High levels of gene expression were observed for *elovl2* transgene in liver and muscle, and reduced mosaicism helped improve DHA and total n-3 PUFAs contents. Potential off-targets effects appeared to result in morphological deformities of transgenic fish, but CRISPR/Cas9-mediated transgenesis of *elovl2* gene in channel catfish can be as a model to demonstrate the feasibility of dramatically enhancing n-3 PUFAs in other aquaculture species.

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Chapter 4 A new strategy for increasing knock-in efficiency: multiple elongase and desaturase transgene knock-in by targeting at long repeated sequences in the channel catfish (*Ictalurus punctatus*) genome

4.1 Abstract

CRISPR/Cas9-mediated knock-in (KI) has a wide application in gene therapy, gene function study and transgenic breeding programs. Unlike gene therapy, which requires accurate KI to correct gene mutation, transgenic breeding programs can accept robust KI as long as integration does not interrupt normal genes functions and result in any negative pleotropic biological effects. High KI efficiency is required to reduce breeding costs and shorten the breeding period, especially, in transferring multiple foreign genes to single individuals. To elevate KI efficacy and achieve multiple genes KIs simultaneously, we introduced a new strategy which enables transgene to integrate into numerous sites of genome by targeting the long repeated sequence (LRS). In a novel use of this simple strategy, for the first time, we successfully generated transgenic fish carrying masu salmon (*Oncorhynchus masou*) *elovl2* gene and rabbitfish (*Siganus canaliculatus*) Δ4 *fad* and Δ6 *fad* genes, and achieved robust target KI of *elovl2* and Δ6 *fad* genes at multiple sites of LRS1 and LRS3, respectively, in the initial generation. This demonstrated that donor plasmid homology arms, nearly identical but not completely the same as the genome sequence, still led to on-target KI. Although target KI efficiencies at LRS1, LRS2 and LRS3 sites were still relatively low in the current study, it is very promising that 100% KI efficiency in the future could be realized and perfected by selection of better LRSs and optimization of sgRNAs.

Keywords: CRISPR/Cas9; knock-in efficiency; long repeated sequence; fatty acids

4.2 Introduction

Transgenesis has boosted the genetic improvement of crops, livestock and fish over the past decades (Ahmad et al., 2012; Maclean and Laight, 2000; Wheeler, 2007). The majority of transgenic studies involved manipulation of single genes. However, many important traits are regulated by interactions of multiple genes on complex metabolic pathways (Johnston et al., 2011). For instance, the synthesis of EPA and DNA from α-linolenic acid (ALA; 18:3n-3) in fish requires desaturation and elongation procedures. Elongase *elovl2* and desaturase Δ4 *fad*, Δ5 *fad* and Δ6 *fad* are all important genes restricting EPA and DHA synthesis in the n-3 fatty acid biosynthetic pathway (Gregory and James, 2014; Li et al., 2010). Overexpression of only one of these genes in transgenic fish improved EPA and DHA contents to a small extent (Alimuddin et al., 2005; 2007; Alimuddin et al., 2008; Cheng et al., 2014; Kabeya et al., 2014). Therefore, modification of n-3 synthetic pathway by knocking-in multiple elongase and desaturase transgenes more thoroughly addressing this complicated biosynthetic pathway may increase EPA and DHA levels greatly. However, multiple genes transgenesis was encumbered by technical limitations of transgenic methods. Co-injection of separate multiple plasmids and transformation with linked transgenes in single vector had low integration efficiency or protein expression using random integration methods in the past (Jankowsky et al., 2001; Lin et al., 2003).

CRISPR/Cas9 system, which includes a complex of Cas9 protein and a single guide RNA (sgRNA), is widely applied in genome editing researches. CRISPR/Cas9 system is efficient in inducing double-strand breaks (DSB) at target site of genome, and then two main mechanisms of DNA break repair, non-homologous end joining (NHEJ) and homology-directed repair (HDR) are responsible for DNA repair, among other DNA-break repair mechanisms, leads to knockout (KO) and knock-in (KI) events. However, unlike KO, KI induced by HDR has low efficiency,

only 1-12.5% in most species (Auer et al., 2014; Hruscha et al., 2013; Li et al., 2019; Wu et al., 2016), limiting its applications in generating transgenic animals.

Therefore, multiple strategies have been explored and applied to increase knock-in efficiency. Double cut by sgRNA increases HDR efficiency by two-fold to five-fold (Zhang et al., 2017). Tild-CRISPR with a PCR-amplified or precisely enzyme-cut transgene donor yielded up to 12 fold higher KI efficiency than HDR in human embryos (Yao et al., 2018). Microhomology-mediated end-joining (MMEJ)-based strategy can improve KI efficiencies up to 20% with short homology arms (5-25 bp), and much higher (up to 10-fold) than HDR-based strategy (Yao et al., 2017b). ssODN (single -stranded oligodeoxynucleotides)-mediated end-joining can achieve 13.3-23.5% precise integration efficiency (Yoshimi et al., 2016), and multiple sgRNAs with overlapping sequences enhanced short ssODN KI efficiency furtherly (Jang et al., 2018). Non-homologous end joining (NHEJ) based strategy also can achieve higher efficiency of robust KI than HDR (Suzuki et al., 2016). In addition, chemicals, such as (3 ‑ (N ‑ benzylsulfamoyl) ‑ 4 ‑ 97 bromo ‑ N ‑ (4 ‑ bromophenyl) benzamide), known as RS-1, can increased KI efficiencies to 26.1% by enhancing HDR (Jayathilaka et al., 2008), five-fold higher than control (Song et al., 2016).

However, KI efficiencies in recent studies were below 27%, which is not high enough for knocking in multiple transgenes with one step. If we assume that every transgene has 27% KI efficiency, then the odds of two transgenes in a founder is only 7.3% (27% × 27%), the probability will decrease to 2.0% (27% × 27%× 27%) for three transgenes KI, and continue to go down dramatically with more transgenes KI. Additionally, it takes a long period to produce transgenic animals by crossbreeding or continuous KI by subsequent generations since some aquaculture fish and livestock need several years to mature. To shorten generation interval and

reduce costs, KI multiple transgenes in one generation strategy with higher efficiency is highly required.

Millions of copies of transposable elements (TE) and other repetitive sequences were detected in eukaryotic genomes. Studies show that 66%-69% of human genome was repetitive or repeat-derived (de Koning et al., 2011), 52.2% of zebrafish genome had repeat content (Howe et al., 2013), and a total of 13.7% repetitive elements were found in channel catfish (Jiang et al., 2011). Long and highly identical repeats are ideal candidate target sequences to achieve high integration rates for transgenesis because single sgRNA with donor DNA is able to be knocked in multiple sites at the same time, improving the chance of robust KI greatly. In this study, we designed multiple plasmids, containing *elvol2* elongase transgene from masu salmon (*Oncorhynchus masou*) and Δ4 *fad* and Δ6 *fad* desaturase transgenes from rabbitfish (*Siganus canaliculatus*), respectively, to target three long and highly identically repetitive sequences in channel catfish genome. We then compared the efficacy of this LRS method with HDR and 2H2OP (two-hit by gRNA and two oligos with a targeting plasmid) KI methods. Our goal was to explore a new strategy to greatly increase KI efficiency to generate transgenic fish carrying multiple transgenes in one step.

4.3 Materials and Methods

4.3.1 Animals

Channel catfish were obtained from the Fish Genetic Research Unit, School of Fisheries, Aquaculture and Aquatic Science at Auburn University, Alabama 36849, USA. The research protocol followed all Standard Operating Procedures (SOP) approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University.

4.3.2 Target sites selection, sgRNAs design and donor plasmids construct

Three different strategies were applied in this study to produce transgenic fish carrying three transgenes, namely, masu salmon *elovl2*, rabbitfish Δ4 *fad* and rabbitfish Δ6 *fad*. In strategy 1, three donor plasmids with 800 bp homology arms were designed to target three different non-coding sites respectively with the HDR mechanism (Ran et al., 2013) (Figure S1). Strategy 2 aimed at knocking in donor plasmids randomly at luteinizing hormone (*LH*), follicle-stimulating (*FSH*) and gonadotropin-releasing hormone 1 (*GnRH1*) genes using the 2H2OP method (Yoshimi et al., 2016) (Figure S2). We also proposed and designed strategy 3, in which three donor plasmids targeted at three LRSs (Figure 8). LRSs were scanned through the whole genome of channel catfish by Red software (Girgis, 2015). Repeats, which were larger than 800 bp, were selected as the primary candidate target sequences. Then intersected repeats with channel catfish genome annotation using bedtools 2.26.0 (Quinlan and Hall, 2010) were removed. The remaining repeats sequences were blasted in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three sequences which had multiple repeats with higher similarity (> 92% percent identify over their entire lengths) were chosen as the final target sequences. The positions of each LRS in channel catfish genome were visualized using chromoMap (Anand and Rodriguez Lopez, 2022). Each LRS has multiple sites in channel catfish genome. Therefore, the strategy can be modeled as follows:

$F = 1 - (1 - p)^x$ (Equation 1)

Where F is the probability that at least one copy of a single donor plasmid integrates with LRS sites in genome. x is the number of LRS sites. We assume that each copy donor plasmid has equal KI chance at all LRS sites, and p is the value of chance. In this study, we tried LRS1 (Figure 8A), LRS2 (Figure 8C) and LRS3 (Figure 8E) as target sequences to design sgRNAs. Theoretically, sgRNA1, sgRNA2 and sgRNA3 with Cas9 protein could recognize and cut 123,

23, and 760 sites (Figure 8B; 8D and 8F), respectively, and achieve donor plasmids KI. The model above could be rewritten as following equations:

$F_{LRS1} = 1 - (1 - p_{LRS1})^{123}$ (Equation 2)

$F_{LRS2} = 1 - (1 - p_{LRS2})^{23}$ (Equation 3)

$F_{LRS3} = 1 - (1 - p_{LRS3})^{760}$ (Equation 4)

Where F_{LRS1}, F_{LRS2} and F_{LRS3} are the KI probability of at least one copy of masu salmon *elovl2*, rabbitfish Δ4 *fad* and rabbitfish Δ6 *fad*, respectively. p_{LRS1}, p_{LRS2} and p_{LRS3} are the probability of KI at single cut site of LRS1, LRS2 and LRS3, respectively. Additionally, we added 80 bp cut sites sequences on the both sides of each donor plasmids to for gRNAs to linearize the circular plasmids *in vivo* that increase KI efficiency further via potential NHEJ mechanisms (Figure 1A, 1C and 1E).

The corresponding sgRNAs were designed by CRISPRscan (Moreno-Mateos et al., 2015) based on scores and location. Three plasmids for LRS method: UBI_masu_salmon_Elvol2, UBI_rabbitfish_Δ4 and UBI_rabbitfish_Δ6 were synthesized by GenScript (Piscataway, NJ). Each plasmid had a plasmid backbone, 80 bp left and 80 bp right cut sequences, 200 bp left and 200 bp right homology arms, zebrafish ubiquitin (UBI) promoter, CDS and terminator (Figure 1). Left and right cut sequences were 80 bp including 40 bp before sgRNA cut site and 40 bp behind sgRNA cut site. UBI promotor sequence was provided by Mosimann (unpublished). CDS sequences were *elovl2* (GenBank: KC847063.1) isolated from masu salmon, Δ4 *fad* (GenBank: GU594278.1) and Δ6 *fad* (GenBank: EF424276.2) desaturase genes isolated from rabbitfish, respectively. The plasmids for HDR and 2H2OP methods were constructed with the same CDS regions as LRS method, but driven by common carp *β-actin* promoter and followed by ocean

pout antifreeze polyA terminator. Additionally, the plasmids for HDR had 800 bp left and 800 bp right homology arms. All plasmids sequences were listed in Figure S3-S8.

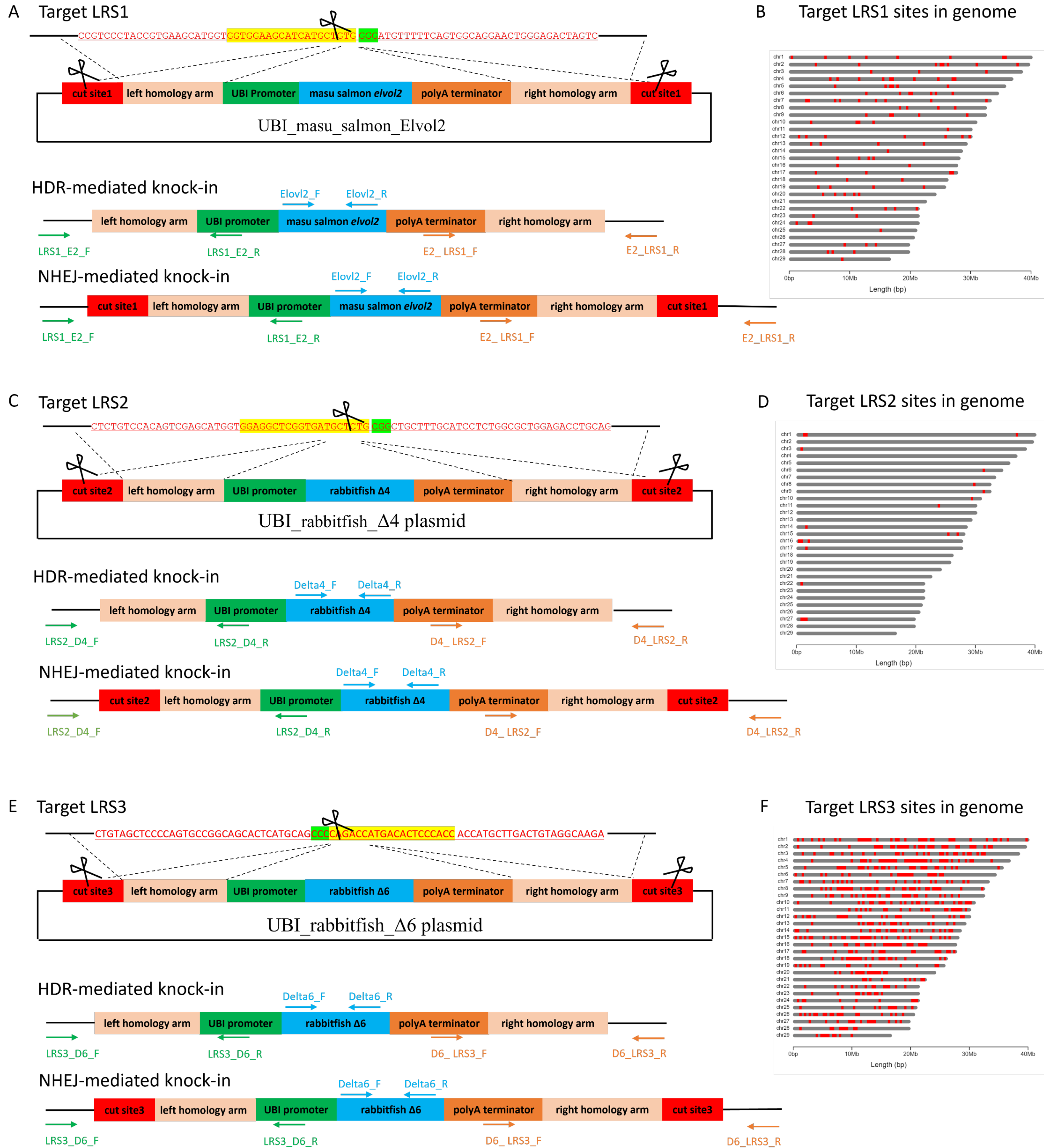


Figure 8. Schematics of HDR- (homology-directed repair-) and NHEJ- (non-homologous end joining-) mediated knock-ins (KIs) of three donor plasmids targeting three long repeated sequences (LRSs) in genome of channel catfish, *Ictalurus punctatus*

(A) HDR- and NHEJ-mediated KI of masu salmon, *Oncorhynchus masou, elovl2* gene targeting LRS1.
(B) Positions of LRS1 cut sites in genome of channel catfish.
(C) HDR- and NHEJ-mediated KI of rabbitfish, *Siganus canaliculatus,* Δ4 *fad* gene targeting LRS2.
(D) Positions of LRS2 cut sites in genome of channel catfish.
(C) HDR- and NHEJ-mediated KI of rabbitfish Δ6 *fad* gene targeting LRS3.
(D) Positions of LRS3 cut sites in genome of channel catfish.

4.3.3 Preparation for sgRNA and Cas9

sgRNA was generated as described by Varshney et al. (2015). Briefly, the sgRNA template was produced by annealing two oligos: a unique oligonucleotide encoding T7 polymerase binding site and the sgRNA target sequence, GGAATTAATACGACTCACTATAGG(N_{20})GT TTAAGAGCTATGCTGG; and a common oligonucleotide (AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC TTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC) encoding the reminder of sgRNA sequence. The sgRNAs were transcribed *in vitro* by Maxiscript T7 Kit (Thermo Fisher Scientific, Waltham, MA), then purified using Zymo RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). The Cas9 protein was obtained from PNA BIO Inc. (Newbury Park, CA).

4.3.4 Microinjection

Sexually mature Kansas strain channel catfish were harvested from the Fisheries Genetics Unit, E.W. Shell Research Center (Auburn University, Auburn, AL). Brood stock spawning followed the procedures of Qin et al. (2016). Microinjection was performed as described by Khalil et al. (2017) with modification. For HDR and LRS methods, approximately 6 nL total mixtures containing approximately 333 ng/μL Cas9 protein, 33 ng/μL each of sgRNAs and 11 ng/μL each of donor plasmids were mixed and co-injected into the yolk of one-cell stage embryos of channel catfish. For 2H2OP method, an extra 11 ng/μL each of short ssODNs were added to the mixture and were co-injected as described above.

4.3.5 Analysis of integration

The mixed fin and barbel clipped samples DNA of six-month-old fingerlings was extracted via proteinase K followed by protein precipitation as described by Kurita at el. (2004). Gene specific primers (Table 7) were designed to detect transgenes in all microinjected fish and other primers were used to detected junction regions. PCR products were analyzed with 1% agarose gel electrophoresis and cloned into the vector of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and transformed into One Shot TOP10F chemically competent *E. coli* (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Colonies were randomly picked for sanger sequencing by Eurofins Genomics (Louisville, KY) .

4.3.6 Transgene expression

Two nine-month-old transgenic fish carrying 3 transgenes were randomly collected and rapidly euthanized. Muscle, liver and Intestine tissues were dissected and flash-frozen in liquid nitrogen. All tissue samples were then sored at – 80 ºC until RNA isolation. Total RNA was isolated from various tissues using RNeasy Plus Universal Mini Kit (QIAGEN, Valencia, CA), and was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol. The expression level of ribosomal 18s mRNA was used as an internal control(Small et al., 2008). The primers used for RT-PCR are listed in Table S1.

4.3.7 Statistical analysis

The association among three transgenes integrations was analyzed with Cochran-Mantel-Haenszel (CMH) test and chi-square test using SAS statistical software (version 9.1, SAS Institute, Inc., Cary, NC). Statistical significance was defined as $P < 0.05$.

4.4 Results

4.4.1 Production of transgenic channel catfish carrying three transgenes

Table 7. Primers used for identification, gene expression and junctions detection of masu salmon *(Oncorhynchus masou) elovl2* gene, rabbitfish (*Siganus canaliculatus)* Δ4 *fad* and Δ6 *fad* genes in transgenic channel catfish, *Ictalurus punctatus*

Primer	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	Description
Elovl2_F	GAGACTCCAGGGTACGAGGA	640	63	Masu salmon elovl2 transgene detection
Elovl2_R	AGGCATCCAACAGGGAAACC			
Delta4_F	TCGCAAGGTTTATAATGTCACCC	770	63	
Delta4_R	ACAGATCCACATAGTCACGGC			
Delta6_F	TGCTTTCCACCCTGACTTGA	710	63	
Delta6_R	AGCAGCACATGTACCGAAGG			
Elovl2_RT_F	GGATGGCTCCTGTTGGACTC	186	63	Reverse transcription-polymerase chain reaction PCR (RT-PCR) of Masou salmon elovl2 transgene
Elovl2_RT_R	TGCCAAGACAAGCTCAACCA			
Delta4_RT_F	GTGGATCTGTCTTGGGCCAT	102	63	
Delta4_RT_R	CCTGGCAAACATCATGAGCG			
Delta6_RT_F	CTCATTGGACCACCGCTTCT	98	63	
Delta6_RT_R	AAGCACCACACCAGATCCAG			
18s_F	GAGAAACGGCTACCACATCC	128	63	Internal control for RT-PCR
18s_R	GATACGCTCATTCCGATTACAG			
LRS1_E2_F	GCCAAACTGAACGATTGGGG	486/726	58	5' and 3' junctions detection
LRS1_E2_R	GCCGTCTGTTGTTTATGTTGCT			
E2_LRS1_F	CAAGGTGCTGTGGTGGTACT	893/1133	58	
E2_LRS1_R	CTCTGGCTGGGACACTCAAG			
LRR2_D4_F	CCTGAATAGAATCCCGCCCA	660/900	58	
LRR2_D4_R	CCGTCTGTTGTTTATGTTGCTGG			
D4_LRR2_F	CGAACACCATTTGTTCCCGAG	916/1156	58	
D4_LRR2_R	TCCCCAATGTCAATATTTAAAGCAA			
LRR3_D6_F	TTCCAGCACTGCCTTAACCC	681/921	58	
LRR3_D6_R	ACTGATCCCTCCATGAGCTT			
D6_LRR3_F	CTCATTGGACCACCGCTTCT	872/1112	58	
D6_LRR3_R	CACAAGAAAGCCCGCAAACA			
NC1_E2_F	CAATGACTGGAGATGGCCCC	970	58	
NC1_E2_R	AAGAGCAAGGCCTAAGCGAT			
E2_NC1_F	TGTTGATGCCTCCATGACCAA	1205	58	
E2_NC1_R	AACGTTGAGAGCAGGTGAGG			
LH_E2_F	TCGATGGTACAGTCCGAGGT	361	58	
LH_E2_R	CATAGTTAAGCCAGCCCCGA			
E2_LH_ F	TGAGATCCAGTTCGATGTAACCC	588	58	
E2_LH_ R	GTCAGTGCCAGCTTCCTCTTT			
GnRH_E2_F	ATGGCTGAGCAGTTACCTGAG	589	58	
GnRH__E2_R	ATAGTTAAGCCAGCCCCGACAC			
E2_ GnRH_ F	AAACGTTCTTCGGGGCGAAA	640	58	
E2_ GnRH_ R	CTTCAACAGTCTCGGGAGGG			
FSH_E2_F	TGGCACCGTACTTCCAGAAC	315	58	
FSH_E2_R	TAGTTAAGCCAGCCCCGACA			
E2_FSH_ F	TTCCCCGAAAAGTGCCACC	431	58	
E2_FSH_ R	AACAACAGCACATCATGCCC			

Specific primers (Table 7) were designed to screen all fish to identify positive fish. One of 160 (0.625%) and 48 out of 214 (22.4%) positive offspring carried masu salmon *elovl2* gene with strategy 1 and 2, respectively, and no Δ4 *fad* and Δ6 *fad* transgenic fish were identified (Table 8). Comparatively, PCR screening results (Figure 9) showed that 25% (46/184), 11.4% (21/184) and 15.2% (28/184) fish were positives for *elovl2,* Δ4 *fad* and Δ6 *fad* transgenes, respectively, using strategy 3 (Table 8).We observed that three transgenes were co-integrated with genome based on Cochran-Mantel-Haenszel (CMH) test (P < 0.001) (Agresti, 2003). Thirteen out of 184 (7%) fish had all three transgenes (Table 9), which were much more than fish carrying two transgenes (4/184) (chi-square test, $P < 0.05$).

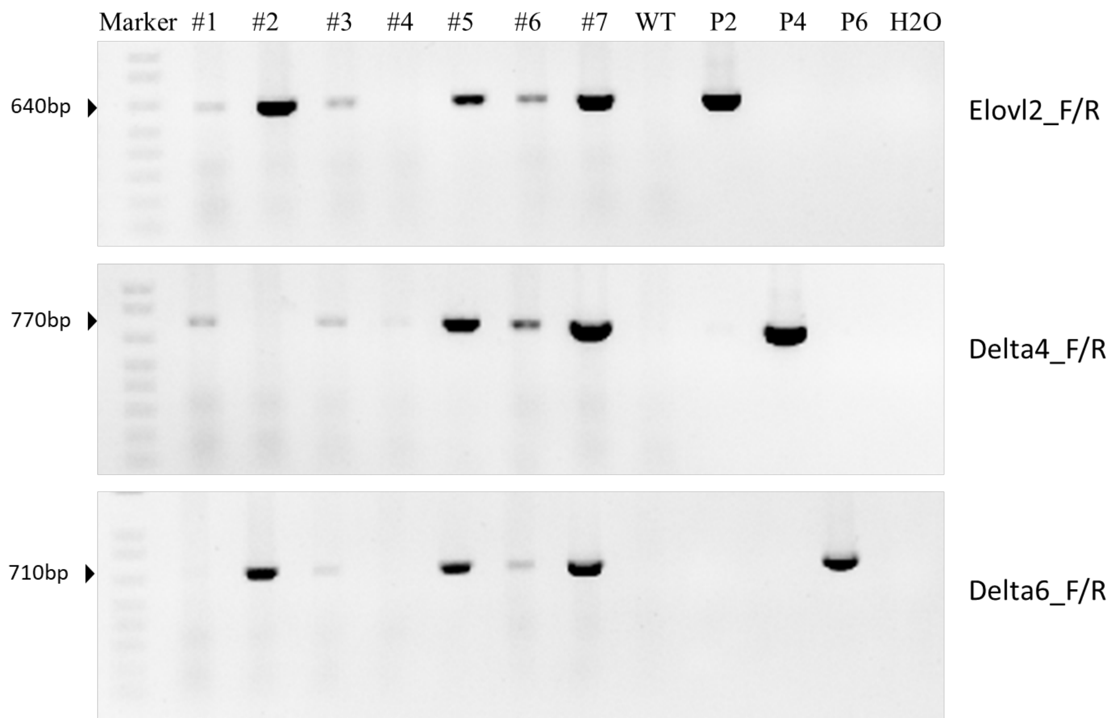


Figure 9. PCR detection of single, double and triple transgene, masu salmon *(Oncorhynchus masou) elovl2* gene, rabbitfish (*Siganus canaliculatus)* Δ4 *fad* and Δ6 *fad* genes, insertions for channel catfish (*Ictalurus punctatus*) targeting long repeated sequences (LRSs). Primers Elovl2_F/R, Delta4_F/R and Delta4_F/R were used to screen for masu salmon *elovl2*, rabbitfish Δ4 *fad* and Δ6 *fad* genes, respectively. WT: wild type; P2: positive control of masu salmon *elovl2*; P4: positive control of rabbitfish Δ4 *fad*; P6: positive control of rabbitfish Δ6 *fad*.

Table 8. Comparison of strategies for multiple genes KIs in channel catfish, *Ictalurus punctatus,* for hatching rate, fingerling survival rate, PCR positive (%) and on-target KI efficiency.

Strategy	Repair Mechanism	Transgene	KI length (bp)	Target locus	Hatching rate	Fingerling survival rate (%)	PCR-positive (%)	On-target KI efficiency(%)
Strategy 1	HDR	*elvol2*	3636	NC1	182/1600 (11.4)	160/182 (87.9)	1/160 (0.63)	0/1 (0)
		Δ4 *fad*	4110	NC2			0/160 (0)	0/0 (0)
		Δ6 *fad*	4104	NC3			0/160 (0)	0/0 (0)
Strategy 2	ssODN-mediated end-joining	*elvol2*	7728	*LH*, *GnRH1* or *FSH*	369/1350 (27.3)	214/369 (58.0)	48/214 (22.4)	0/48 (0)
		Δ4 *fad*	8202	*LH*, *GnRH1* or *FSH*			0/214 (0)	0/0 (0)
		Δ6 *fad*	8196	*LH*, *GnRH1* or *FSH*			0/214 (0)	0/0 (0)
Strategy 3	HDR or NHEJ	*elvol2*	2437	LRS1	635/3718 (17.1)	184/635 (29.0)	46/184 (25)	6/46 (13.0)
		Δ4 *fad*	2911	LRS2			21/184 (11.4)	0/21 (0)
		Δ6 *fad*	2905	LRS3			28/184 (15.2)	5/28 (17.9)

Note: HDR, homology-directed repair; ssODN, single-stranded oligodeoxynucleotide; NHEJ, non-homologous end joining; *elovl2* gene isolated from masu salmon (*Oncorhynchus masou)*; Δ4 *fad* and Δ6 *fad* transgenes isolated from rabbitfish (*Siganus canaliculatus)*; NC1,2 and 3, Non-coding region 1 (GenBank: NC_030416.1, 19128961-19129800), 2 (GenBank: NC_030417.1, 23338205-2338224) and 3 (GenBank: NC_030422.1, 12760701-12760720) in channel catfish genome; *LH*, luteinizing hormone; *FSH*, follicle-stimulating; *GnRH1*, gonadotropin-releasing hormone 1; LRS1, 2 and 3, long repeated sequences in channel catfish genome

Table 9. Percentage of single, double and triple transgene, masu salmon *(Oncorhynchus masou) elovl2* gene, rabbitfish (*Siganus canaliculatus)* Δ4 *fad* and Δ6 *fad* genes, insertions for channel catfish (*Ictalurus punctatus*) targeting long repeated sequences (LRSs)

Transgenes	Only *elovl2*	Only Δ4 *fad*	Only Δ6 *fad*	*Elovl2* and Δ4 *fad*	*Elovl2* and Δ6 *fad*	Δ4 and Δ6 *fad*	*Elovl2*, Δ4 and Δ6 *fad*	total
Number	25	4	11	4	4	0	13	184
Percentage	13.6%	2.2%	6.0%	2.2%	2.2%	0	7%	

4.4.2 KI efficiency enhancement by targeting LRSs

To investigate KI efficiency of three strategies, we ran PCR to detect the 5' upstream and 3' downstream junction regions of all positive fish. Surprisingly, no perfectly precise KI event was detected in positive fish produced by strategy 1 and strategy 2 in this study. In strategy 3, we found zero Δ4 *fad* on-target KI fish, but six of the *elovl2* transgene correctly targeted KI fish and five Δ6 *fad* on-target KI fish as expected, of which three fish had both *elovl2* and Δ6 *fad* on-

target KI based on PCR of junctions (Figure 10A and 10B) and sequencing data. Sequencing data was aligned with predicted perfectly on-target KI sequences (Figure S3-S8) using MAFFT (version 7, https://mafft.cbrc.jp/alignment/server/) and blasted against channel catfish genomes (GenBank: GCA_001660625.2 and GCA_004006655.3) using NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Actual target sites in chromosomes of genome were determined according to the sequences with lowest E-value.

We found that donor plasmids masu salmon *elovl2* and rabbitfish Δ6 *fad* inserted in multiples sites of LRS1 and LRS3 successfully (Figure 10C and 10D). Donor of *elovl2* transgene integrated into 15 locations of genome, which were more than four locations of Δ6 *fad* integration under sequencing the limited number of TA clones. Both HDR and NHEJ mediated KI were detected in this study. HDR mediated KI occurred in both 5' and 3' junctions, while NHEJ mediated KI was only identified at the 5' upstream junction. There were many deletions, insertions and point mutations around the 5' and 3' junctions. As sgRNAs targeted at multiple sites of LRSs, the homology arms flanking each target sites of genome were not completely the same as homology arms of donor plasmids. We identified two patterns of homology recombination based on sequencing (Figure 10E). One pattern was that homology arms sequences of genome were reserved and only functional regions of donor plasmids, such as promoter, transgene and polyA, were integrated with the genome. The other one is literal integration of the functional elements from the donor plasmids with homology arms inserted in the genome.

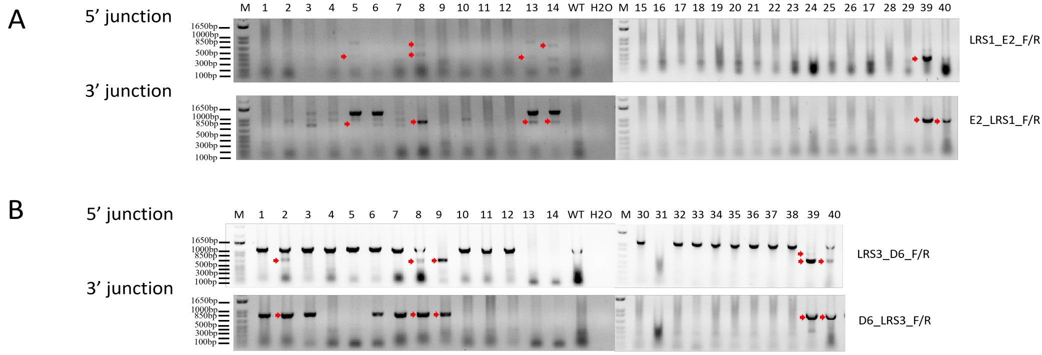
4.4.3 Transgenes expression

We sacrificed two transgenic fish carrying all three transgenes, extracted total RNA from muscle, liver and intestine, then ran reverse transcription-polymerase chain reaction PCR (RT-

A

5' junction

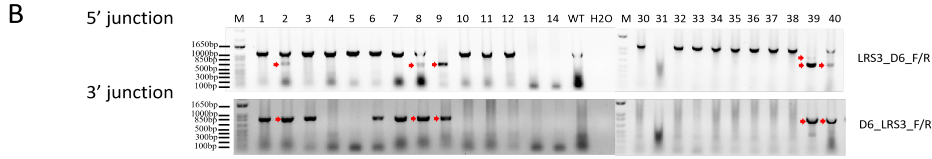
3' junction



B

5' junction

3' junction



C

5' junction

HDR:

GenBank	5' upstream location	5' upstream sequence / left homology arm / UBI promoter	
NC_030419	chr4: 23146532..23146709	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	Reference
NC_030416	chr1: 10744860..10745036	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030416	chr1: 29851459..29851634	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//CCACC---------------------TGAAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	-21
NC_030416	chr1: 30301184..30301369	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030418	chr3: 19892431..19892806	GCCAAACTGA//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030419	chr4: 23146532..23146709	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030419	chr4: 23146532..23146709	GCCAAACTGA//CTGTG--------------TGTCA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	-14
NC_030419	chr4: 23146532..23146709	GCCAAACTGA//CAGCG------TGTGG//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	-6
NC_030420	chr5: 25382576..25382752	GCCAAACTGA//AGACGGAAGGCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030425	chr10: 10865726..10865894	GCCAAACTGA//AGACGGAAGCCACTCTTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	pm1
NC_030425	chr10: 28093214..28093391	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030426	chr11: 10763925..10764102	GCCAAACTGA//AGACGGAAGCCACTCCTCAGT---------------------------------AAAAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	-33
NC_030427	chr12: 18518952..18519128	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030427	chr12: 18518952..18519326	GCCAAACTGA//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030432	chr17: 10184825..10185006	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030441	chr26: 18440600..18440997	GCCAAACTGA//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030441	chr26: 18440820..18440997	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NW_016098006	IpCoco_scf00653: 50933..51105	GCCAAACTGA//AGACGGAAGCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
PKUT01000270	yyscf_270: 2497448..2497823	GCCAAACTGA//GCATCATGCTACCAGCAAAGTTCTAGAATT	0

NHEJ:

	5' upstream location	5' upstream sequence / right cute site:40bp / left homology arm / UBI promoter	
NC_030419	chr4: 23146532..23146909	GCCAAACTGA//GGTGGAAGCATCATGCTGTGGGG//TAGTCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	Reference
PKUT01000008	chr8: 29635368..29635720	GCCAAACTGA//GGTGGAAGCATCATGCTGTGGGG//TAGTCCACTCCTCAG//GCATCATGCTACCAGCAAAGTTCTAGAATT	0

3' junction

HDR:

GenBank	3' downstream location	masu salmon *elovl2* / ploy A / right homology arm / 3' downstream sequence	
NC_030419	chr4: 23147110..23147141	CAAGG//AGTAGGATCC//AAGTTGTGGG//ACAACTCTGTGAATGTCCTTGAGTGTCCCAGCCAGAG	Reference
NC_030419	chr4: 23146910..23147141	CAAGG//AGTAGGATCC//AAGTTGTGGG//TGAAG//GGACCCTAAGAAT//AAACGAGTGGCTACAGGACAACT//GTCCCAGCCAGAG	pm7
NC_030419	chr4: 23146910..23147141	CAAGG//AGTAGGATCC//AAGTTGTGGG//TCCA-CAGGAACA//AAAGGACAACTCCGATAGCTAC//CAACCTGC//TCCCAGCCAGAG	-1, +1, +8, pm4
NC_030419	chr4: 23146910..23147141	CAAGG//AGTAGGATCC//AAGTTGTGGG//CTCAT//TCAAGATAACAA//TGGCT//ACTCTGGGAATGTCCTTGAGTGT-CCAGCCAGAG	-1, +1, pm5
NC_030419	chr4: 23146910..23147141	CAAGG//AGTAGGATCC//AAGTTGTGGG//AAAGGAGTGGCTACAGGACAACT//GTCCCAGCCAGAG	pm2
NC_030419	chr4: 23146910..23147141	CAAGG//AGTAGGATCC//AAGTTGTGGG//AAAGGGGTGGCTACAGGACGACT//GTCCCAGCCAGAG	pm4
NC_030422	chr7: 7513884..7514027	CAAGG//AGTAGGATCC//AAGTTGTGGG//TCTGTGAATGTCCTTGAGTGTCCCAGCCAGAG	0
NW_016102693	IpCoco_scf05505: 135..367	CAAGG//AGTAGGATCC//AAGTTGTGGG//ATGTA--GAGAC//TCTGTGAATGTCCTTGAGTGTCCCAGCCAGAG	-2

D

5' junction

HDR:

GenBank	5' upstream location	5' upstream sequence / left homology arm / UBI promoter	
NC_030442	chr27: 12081762..12081948	TTCCAGCACT//TGGAGACATGCTTGGCCAGT//GCATCATGCTACCAGCAAAGTTCTAGAATT	Reference
NC_030417	chr2: 30520347..30520512	TTCCAGCACT//TGGAGACATGCTTGGCCAGT//GCATCATGCTACCAGCAAAGTTCTAGAATT	0
NC_030437 or NC_030442	chr22: 6464124..6464510 or chr27: 12081762..12082148	TTCCAGCACT//AGAGATTCAC//CCACCTTCCATTTGAGGA//GCATCATGCTACCAGCAAAGTTCTAGAATT	pm2
NC_030433	chr18: 10249927..10250109	TTCCAGCACT//TGGAGACATGCTTGGCCAGT//GCATCATGCTACCAGCAAAGTTCTAGAATT	0

NHEJ:

	5' upstream location	5' upstream sequence / right cute site:40bp / left homology arm / UBI promoter	
NC_030442	chr27: 12081762..12082148	TTCCAGCACT//CAGCCCCAGACCATG//AAGACTTGGCCAGT//GCATCATGCTACCAGCAAAGTTCTAGAATT	Reference
NC_030424	chr9: 5032738..5033146	TTCCAGCACT//GTTG--A---GAGTCA//CAGCCCCAGACCATG//AAGACTTGGCCAGT//GCATCATGCTACCAGCAAAGTTCTAGAATT	-5, pm1

3' junction

HDR:

GenBank	3' downstream location	rabbitfish Δ6 / ploy A / right homology arm / 3' downstream sequence	
NC_030442	chr27: 12082349..12082351	CCTTC//AATGAGATCC//AAGTTACCAT//TGTTTGCGGGCTTTCTTGTG	Reference
Unknown	Unknown	CCTTC//AATGAGATCC//AAGTTACCAT//TGTTTGCGGGCTTTCTTGTG	0
Unknown	Unknown	CCTTC//AATGAGATCC//AAGTTACCAT//TGTCTCTGTA//ACATGATTCC//TGTTTGCGGGCTTTCTTGTG	pm2
Unknown	Unknown	CCTTC//AATGAGATCC//AAGTTACCAT//CACAGGGCATGG//TGTTTGCGGGCTTTCTTGTG	pm1

E

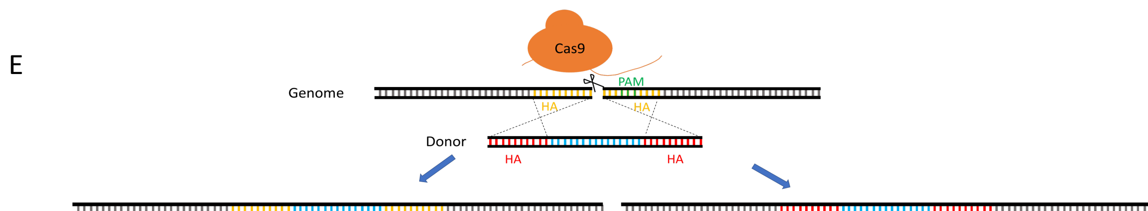


Figure 10. Knock-in (KI) of masu salmon *(Oncorhynchus masou) elovl2* and rabbitfish (*Siganus canaliculatus)* Δ6 *fad* genes into long repeated sequences (LRS) of channel catfish (*Ictalurus punctatus*) genome
(A) PCR detection of 5' and 3' junctions after KI of masu salmon *elovl2* transgene into LRS1. Primers LRS1_E2_F/R and E2_LRS1_F/R were used for 5' and 3' junctions, respectively. Red arrows indicates KI bands. WT, wild type control; H2O, negative water control. Same as below.
(B) PCR detection of 5' and 3' junctions after KI of rabbitfish Δ6 *fad* transgene into LRS3. Primers LRS3_D6_F/R and D6_LRS3_F/R were used for 5' and 3' junctions, respectively. Red arrows indicate KI bands.
(C) Sanger sequencing results of PCR fragments amplified from the junctions after KI of masu salmon *elovl2* transgene into LRS1. HDR indicated homology-directed repair mediated KI; NHEJ indicated non-homologous end joining mediated KI; '-' represents deletion; '+' represents insertion; pm represents point mutation. Same as below.
(D) Sanger sequencing results of PCR fragments amplified from the junctions after KI of rabbitfish Δ6 *fad* transgene into LRS3. Unknown indicates no precise locations were found according to sequencing results.
(E) Schematics of homology arms recombination after HDR-mediated KI. Homology arms originated from either genome or donor DNA were preserved after HDR-mediated KI. HA, homology arms.

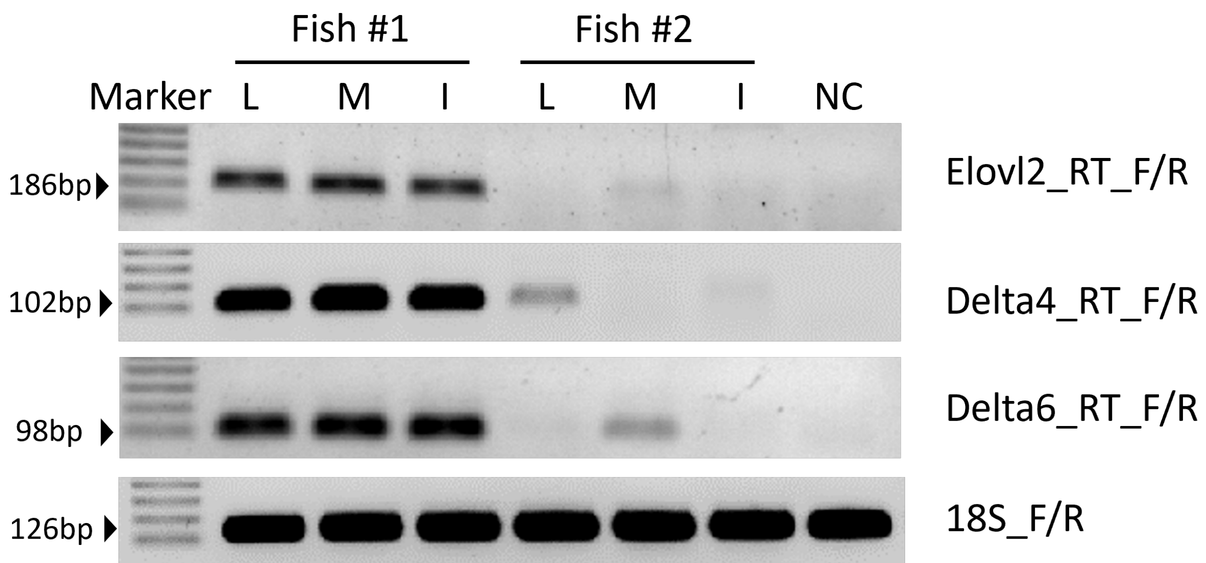


Figure 11. Detection of masu salmon *(Oncorhynchus masou) elovl2*, rabbitfish (*Siganus canaliculatus)* Δ4 and Δ6 transgene expression in triple transgenic channel catfish (*Ictalurus punctatus*) utilizing RT-PCR. L: liver; M: muscle, I: intestine; NC: non-transgenic control. Primers Elovl2_RT_F/R, Delta4_RT_F/R and Delta6_RT_F/R were used to detect masu salmon *elovl2*, rabbitfish Δ4 and Δ6 gene expression, respectively. 18S RNA as an internal control was amplified by primers 18S_F/R.

PCR) with gene specific primers (Table 7) to analyze gene expression levels of masu salmon *elovl2*, rabbitfish Δ4 *fad* and rabbitfish Δ6 *fad* transgenes in channel catfish. Results showed that all three transgenes had gene expressions in three tissues (Figure 11). Transgenes expression levels varied from fish to fish. All three transgenes had high gene expressions in fish #1, but fish #2 only had gene expressions of *elovl2* and Δ6 *fad* in muscle, and Δ4 *fad* transgene expression in liver tissue.

4.5 Discussion

In this study, we produced transgenic channel catfish carrying masu salmon *elovl2*, rabbitfish Δ4 *fad* and Δ6 *fad* transgenes, aiming at modifying n-3 PUFAs biosynthesis pathways to improve EPA and DHA levels. Three CRIPSR/Cas9 strategies were performed to KI these transgenes at one step. Theoretically, three different sgRNAs targeted at three genome sites respectively to generate DSBs in strategy 1 and 2, leading to HDR mediated KI (Wang et al., 2013) or ssODN-mediated end joining (Yoshimi et al., 2016) KI. We observed masu salmon *elovl2* transgene was transferred into channel catfish, but the other two transgenes were not detectable in strategy 1 and 2, suggesting the masu salmon *elovl2* gene was more amenable to gene transfer than the rabbitfish desaturase genes. Previous studies demonstrated successes of HDR mediated and ssODN-mediated end joining KI of single transgene (Paix et al., 2015; Yao et al., 2017a; Yoshimi et al., 2016). Although none of the three transgenes were precisely integrated into expected target sites in this study, we confirmed that three fish had both *elovl2* and Δ6 *fad* on-target KI based on PCR of junctions. The efficiency of KI via HDR is low due to constraint of the competing NHEJ pathway (Chu et al., 2015; Maruyama et al., 2015). Multiple sgRNAs amplified chance of NHEJ and off-target risk (Fortin et al., 2019). Additionally, mixture of multiple sgRNAs and donor plasmids greatly decreased the concentrations of each component

of CRISPR/Cas9 system, might result in low efficiency of sgRNAs activity (Ren et al., 2014). Comparatively, we produced transgenic fish carrying three transgenes in the initial P_1 generation by targeting LRSs. The double or triple transgenes were co-integrated and exerted synergistic effects, which also was observed in previous studies with random integration (Jankowsky et al., 2001; Langenau et al., 2008) .

We also achieved precise KI of masu salmon *elovl2* and rabbitfish Δ6 *fad* genes at LRS1 and LRS3, respectively, with strategy 3. LRS is the high homology sequence which allows a single sgRNA to target at multiple sites (Yang et al., 2017). Targeting at LRS increased the probability of DSB proportionately to the number of a single sgRNA target sites. LRSs of sufficient length made it possible for us to incorporate and design 200 bp as right and left homology arms in donor plasmids. Our sequencing results revealed that both masu salmon *elovl2* and rabbitfish Δ6 *fad* genes were precisely integrated into multiple loci of the multiple chromosomes, which could result in these two transgenes harboring multiple copies in a single cell. Transgene expression is usually associated with copy numbers. Too many copy numbers of gene integration might lead to gene silencing in plants (Tang et al., 2006). However, four to five copies of integrated transgenes stably expressed the protein product (Kohli et al., 1999). Multiple copies of transgene also increased the gene expression in animals (Kong et al., 2009). Additionally, expression levels of transgene are greatly dependent on the chromatin structure surrounding the integration site, producing chromosome position effects (Karpen, 1994). We observed that all three transgenes had co-expression in this study, but the gene expression levels varied from fish to fish, which might due to differences in KI copies, mosaicism (Mehravar et al., 2019), or position effects (Dobie et al., 1996).

In previous studies, left and right homology arms of donor plasmids were always the same as sequences in genome (Bai et al., 2020; Ran et al., 2013), achieving perfect insertion after homologous recombination. While, we first demonstrated that using homology arms that were highly identical but not completely the same as the genomic sequences, on-target KI was still accomplished. Large deletions, random insertions and point mutations only occurred around homology arms regions after HDR, which seemed not affect the functional regions of transgene. In addition, we observed either homology arms originated from donor plasmids or genomic sequences were preserved after insertions as crossover and non-crossover homologous recombination pathways (Krejci et al., 2012). Beside HDR-based KI, NHEJ-based KI occurred in this study since two cut site sequences were added to flanks of donor plasmids, demonstrating that combination of NHEJ and HDR could have potential application in enhancement of CRISPR/Cas9-mediated KI (Yoshimi et al., 2021).

Although we achieved KI of two transgenes by targeting at LRSs, which was better than the other two strategies, the efficiencies were much lower than our expectation, and three genes KI with two on-target. Theoretically, we could obtain over 90% overall KI efficiency according to the model even if only 10% KI probability existed for each site. In fact, there were only 13.0%, 0% and 17.9% overall on-target KI efficiency in this study. It indicated that KI efficiencies of each site were extremely low in this study, which might be due to inapplicable sgRNAs design (Doench et al., 2016; Ren et al., 2014) or inappropriate concentrations of CRISPR/Cas9 components (Simora et al., 2020) or unknown functions in some of the uncertain regions in the LRS that resist alteration or other immune protective functions of the catfish. Optimization of CRISPR/Cas9 to increase KI efficacy at each site will be required in the future. Meanwhile, advanced understanding of the catfish genome and its regulative elements with the

omics technologies would benefit the selection of the best LRS. To our knowledge, our current report is so far the first documentation on selecting LRSs, non-coding repetitive sequences as target sites to reduce disruption of genes functions and achieve multiple transgenes KI. However, non-coding RNA and DNA could have important functions as structural, regulatory or catalytic molecules (Shabalina and Spiridonov, 2004). Thus, we will evaluate the effects of targeting LRSs on fish growth, development and reproduction at the next step. In addition, given there are many more LRSs as target candidates in channel catfish genome, developing a tool to look for better target LRSs to achieve almost 100% of KI efficiency is our long-term goal.

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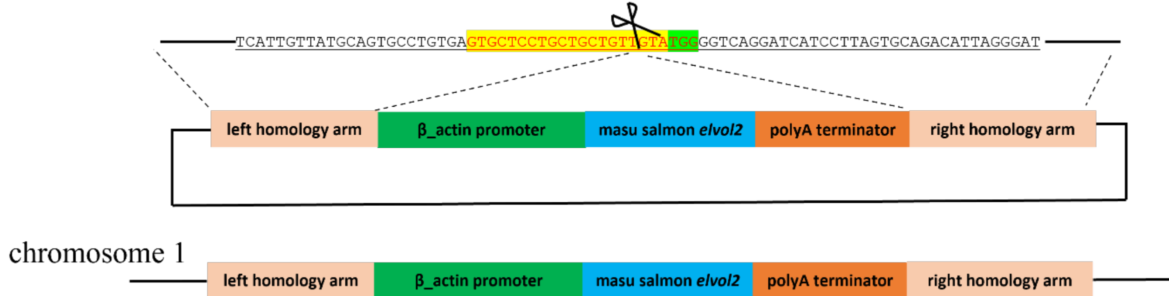
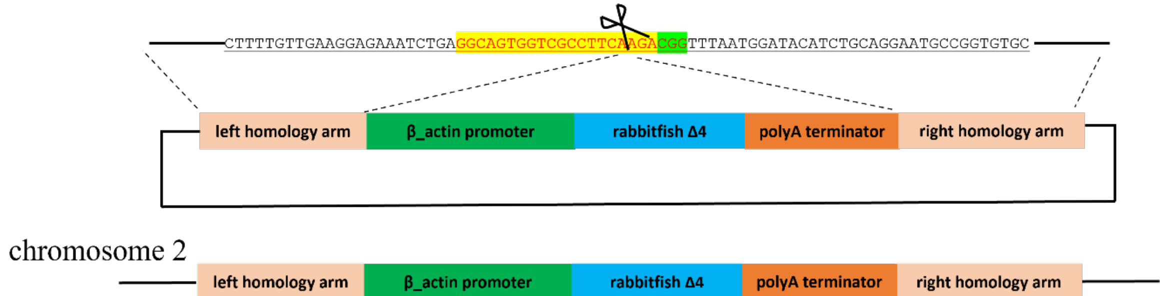
Appendix A

Supplementary information

A Target at chromosome 1 non-coding region



B Target at chromosome 2 non-coding region



C Target at chromosome 7 non-coding region

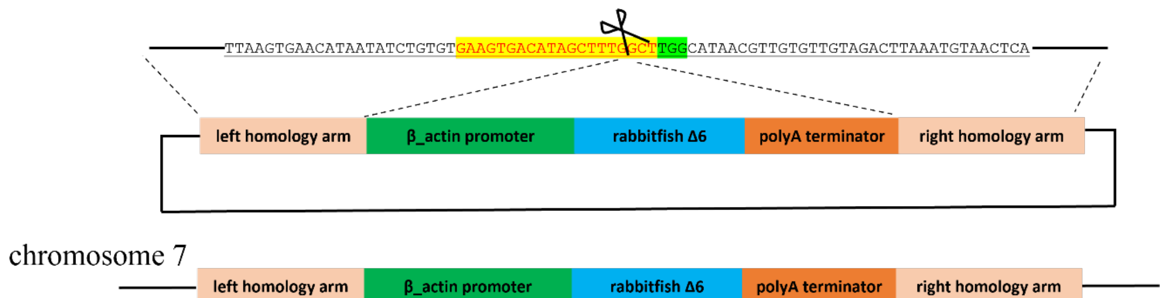


Figure S1. Schematics of HDR- (homology-directed repair-) mediated KIs of three donor plasmids driven by common carp β actin promoter targeting three non-coding regions, respectively, in genome of channel catfish, *Ictalurus punctatus*

(A) KI of masu salmon, *Oncorhynchus masou, elovl2* gene targeting non-coding region in chromosome 1 (GenBank: NC_030416.1, 19128961-19129800).

(B) KI of rabbitfish, *Siganus canaliculatus,* Δ4 *fad* gene targeting non-coding region in chromosome 2 (GenBank: NC_030417.1, 23338205-2338224).

(C) KI of rabbitfish Δ6 *fad* gene targeting non-coding region in chromosome 7 (GenBank: NC_030422.1, 12760701-12760720).

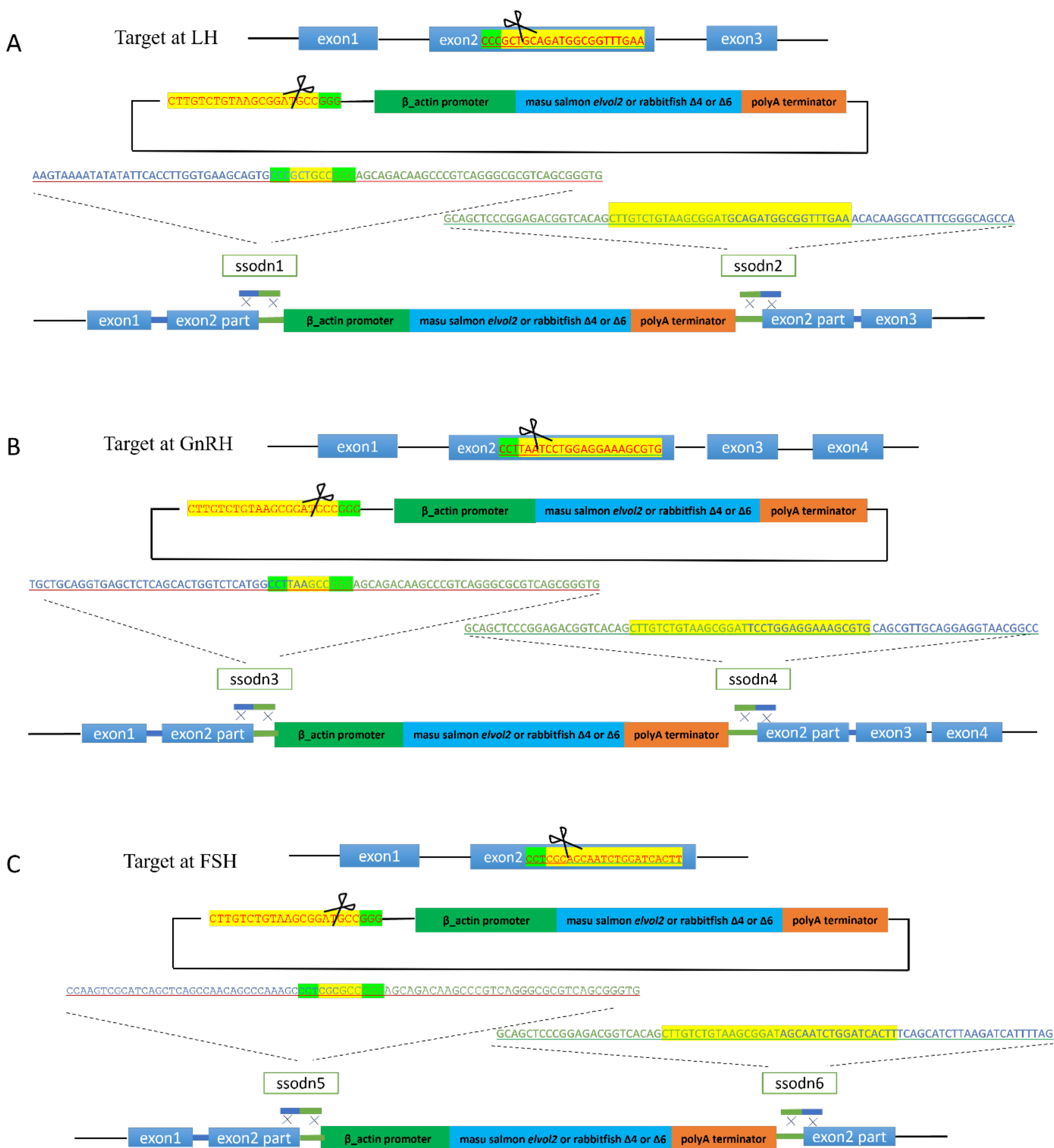


Figure S2. Schematics of ssODN-mediated end-joining KIs of masu salmon *(Oncorhynchus masou) elovl2* gene, rabbitfish (*Siganus canaliculatus)* Δ4 *fad* and Δ6 *fad* genes driven by common carp β actin targeting *LH* (luteinizing hormone), *FSH* (follicle-stimulating hormone) and *GnRH1* (gonadotropin-releasing hormone 1) in channel catfish, *Ictalurus punctatus*. ssodn: single -stranded oligodeoxynucleotides.
(A) KIs of masu salmon *elovl2* or rabbitfish Δ4 *fad* or Δ6 *fad* gene targeting *LH*.
(B) KIs of masu salmon *elovl2* or rabbitfish Δ4 *fad* or Δ6 *fad* gene targeting *GnRH1*.
(C) KIs of masu salmon *elovl2* or rabbitfish Δ4 *fad* or Δ6 *fad* gene targeting *FSH* .

GTGTGTGTATATACAGTGCATCCGGAAAGTATTCACAGCGCTTCACTTTTCCCACATTTTGTTATGTTA

CAGCCTTATTCCAAAATGGATTAAATTCATTATTTTCCTCAAAATTCTACAAACAATACCCCATAATGA

CAATGTGAAAGAAGTTTGTTTGAAATCTTTGCAAATTTATTAAAAATAAAAAACAAAAAAAGCACATG

TACATAAGTATTCACAGCCTTTGCCATGACACTCAAAATTGAGCTCAGGTGCATCCTGTTTCCACTGAT

LRS1

CATCCTTGAGATGTTTCTACAACTTGATTGGAGTCCACCTGTGGTAAATTCAGTTGATTGGACATGATT

TGGAAAGGCACACACCTGTCTATATAAGGTCCCACAGTTAACAATGCATGTCAGAGCACAAACCAAGC

CATGAAGTCCAAGGAATTGTCTGTAGACCTCCGAGACAGGATTGTATCGAGGCACAGATCTGGGGAA

GGGTACAGAAACATTTCTGCAGCATTGAAGGTCCCAATGAGCACAGTGGCCTCCATCATCCGTAAATG

LRS1_E2_F

GAAGACGTTTGGAAGCACCAGGACTCTTCCTAGAGCTGGCCGTCCGGCCAAACTGAACGATTGGGGG

AGAAGGGCCTTAGTCAGGGAGGTGACCAAAAACCCGATGGTCACTCTGACAGAGCTCCAGCGTGTCTC

TGTGGAGAGAGGAGAACCTTCCAGAAGAACAACCATCTCTGCAGCACTCCACCAATCAGGCCTGTATG

GTAGAGTGGCCAGACGGAAGCCACTCCTCAGTAAAAGGCACATGAAAGCCCACCTGGAGTTTGCCAA

AAGGCACGTGAAGGACTCTCAGACCAAAGATTGAACAAAGATTGATCTCTTTGGCCTGAATGGCAAGT

left homology arm

GTCATTTCTTGAAGAAAACCAGGCACAACTCATCACCTGGCCAATACCGTCCCTACCGTGAAGCATGG

TGGTGGAAGCATCATGCTACCAGCAAAGTTCTAGAATTTGTCGAAACATTTATGTTATATATTTCCTGA

LRS1_E2_R

AAAAAATTCTGAGTAAGTTCTTAAGTGTTATTGCCAGCAACATAAACAACAGACGGCAAAATGAATAA

ATGATAACAAAGCAGTAGGCTTAAATAAACCTAATTTTTATAGGCTGTTCTCTACAACCCTCAAACAG

TGATTAGTTTTGTACTTATAAACTTGCCCTTTCATTCATATTTCAAGAAAATTGGTTCAGAAGATCTGG

ATATTCTAGCAGTTGTTCAAGCTCATGGAGGGATCAGTGACCTGATTCCAAATGACTAGGCCTAATCC

AGAAATTAGATGACTGTCAACATAAAAAGGCACAGCACTCACTAGCTGCCCTATATATTTTATTATATT

TTACATATATTATTTTATTTATTTAGCTCTGAGTGCTGTACTTTCTGGTTAAAGAAAACTGCTTACAACA

GCTAACCTGTACTACCTCAGGCTCAGGGAATTTGGAACAGGTTTGTCTGGTTTGTTTCTTTAACCATGC

ATGCTTGTTTTCAACTATGGCAACACAGTCACATGGGACATTACAGAAATGATTTGTCGATGACATGC

GACTTTTCTTTAATAAAGCGCAAAGATCCCAAAAAGCAAACTTTTAACAAAAATCATATAATTATATTT

TCAATCCAGCTTTGTAGCAACTTTGTGCTGCTGTTCACTCAGCAACAGATAGTCAGTATAAGGTCAGTG

UBI promoter

TGTCTCAAAGCAGTGCCATCTGTTTCACACATTGCGTTCTATATATAAGTGTGCTGGTTGACACGACAC

TGTATAAGGCCTAGGCTAAAACACAAACAATGTAGAATGACACTGTGTTTTTTTTGTAAACAAATGTT

GTTTTTGGTTAAACATCTTTGTGAAAACATCCTCCTGTCATGTATTTGCTATATTCAAATGTTAAACCCG

TGCAGAATAGAACATATACAAAAAAAAACAACACAACACATTTTTAAACATTATTAAATATCAAGTAT

TGCTGGCAGTTCTGTTTCTGTTTTACAGTACCCTTTGCCACAGTTCTCCGCTTTTCCTGGTCCAGATTCC

ACAAGTCTGATTCACCAATAGCAAAGCGAATAAACAACCAAAGCAGCCAATCACTGCTTGTAGACTGT

CCTGCGAGACCGGCCCATTCCAGCACATTCTGGAAACTTCCTTTATATGATAATTATAAATACATTTAA

ATTATTGATACAAAACATGTAATTCCTAGAACATAACCATAGCAATCATTAGTTTTCAGGGTAATTATG

TATTTTTAGGATTTGACTGCGGAAAGATCTGGTCATGTGACGTCTCATGAACGTCACGGCCCTGGGTTT

CTATAAATACAGTAGGACTCTCGACCATCGGCAGATTTTTCGAAGAAGAAGATCAGTTTCAGGAGCCG

TACTGTTCCGTTATGAACCATTTACAAAGTTTGGACGAGCGGTTAAACGCGCTTTTTACTTCCTGTTTG

Elovl2_F

AAGACAGAGACTCCAGGGTACGAGGATGGCTCCTGTTGGACTCATATCTCCCCACCCTCTCCCTCACC

ATCCTTTACCTCCTCACAGTATACCTGGGATCAAAATACATGAGGAACAGGCCAGCATACTCACTCAA

GGGGGTCTTACAAGTGTACAACTTCTCTGTGACCATGCTCTCCCTGTACATGCTGGTTGAGCTTGTCTT

GGCAACCTTGTCGGCAGGCTACCGTCTGCAGTGTCAGGGACTTCACGAGGCAGGGGAGGCCGACCTCA

E2_LRS1 _F

GGGTAGCCAAGGTGCTGTGGTGGTACTACTTCTCCAAGGTGATTGAGTTCCTGGACACCATCTTCTTTG

TGCTGCGGAAGAAGAACAGCCAGATCACCTTCCTGCACGTTTATCACCACGCCTCCATGTTCAACATTT

GGTGGTGTGTTCTGAACTGGATCCCCTGTGGACAGAGTTTCTTTGGGCCGACTCTTAACAGTTTCATCC

Masu salmon *elovl2*

ATGTGTGTATGTACTCCTACTATGGCCTGTCCACTATACCCTCCATGCAGAAATACCTCTGGTGGAAGC

GCTACCTGACTCAGGCTCAGTTGATCCAGTTTATACTGACCATCACACACACCCTGTCTGCAATCGTCG

Elovl2_R

TCCCTTGTGGTTTCCCTGTTGGATGCCTGCTCTTCCAGTTCTCCTACATGGCCACCCTCGTCATCCTTTTT

GTCAACTTCTACGTCCAGACCTACAGAAAAAGACGGCCAGAAGAATCCATCAAGTCCAGTCGCCCAA

ATGGCCACTCTGTCTCCACCAATGGCACCAGCTTCAAGAAGAGAAAGTAGGATCCAGACATGATAAG

Poly A

ATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTT

GTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTGTGGGGATGTTTTTCAGTG

GCAGGAACTGGGAGACTAGTCAGGATCAAGGGAAAGATGAATGCAGCAATGTAGAGACATCCTTGAT

right homology arm

GAAAACCTGCTCCAGAGCGCTCTGGACCTCAGACTGGGGCGAAGGTTCATCTTCCAACAGGACAATGA

LRS1

CCCTAAGCACACAGCCAAGATAACGAAGGAGAGGCTACAGGACAACTCTGTGAATGTCCTTGAGTGT

E2_LRS1 _R

CCCAGCCAGAGCCC

Figure S3. Sequence at LRS1 after insertion of the UBI_masu_salmon_*elovl2* donor plasmid with perfect HDR-mediated knock-in

GTGTGTGTATATACAGTGCATCCGGAAAGTATTCACAGCGCTTCACTTTTCCCACATTTTGTTATGTTA

CAGCCTTATTCCAAAATGGATTAAATTCATTATTTTCCTCAAAATTCTACAAACAATACCCCATAATGA

CAATGTGAAAGAAGTTTGTTTGAAATCTTTGCAAATTTATTAAAAATAAAAAACAAAAAAAGCACATG

TACATAAGTATTCACAGCCTTTGCCATGACACTCAAAATTGAGCTCAGGTGCATCCTGTTTCCACTGAT

CATCCTTGAGATGTTTCTACAACTTGATTGGAGTCCACCTGTGGTAAATTCAGTTGATTGGACATGATT

TGGAAAGGCACACACCTGTCTATATAAGGTCCCACAGTTAACAATGCATGTCAGAGCACAAACCAAGC

LRS1

CATGAAGTCCAAGGAATTGTCTGTAGACCTCCGAGACAGGATTGTATCGAGGCACAGATCTGGGGAA

GGGTACAGAAACATTTCTGCAGCATTGAAGGTCCCAATGAGCACAGTGGCCTCCATCATCCGTAAATG

LRS1_E2_F

GAAGACGTTTGGAAGCACCAGGACTCTTCCTAGAGCTGGCCGTCCGGCCAAACTGAACGATTGGGGG

AGAAGGGCCTTAGTCAGGGAGGTGACCAAAAACCCGATGGTCACTCTGACAGAGCTCCAGCGTGTCTC

TGTGGAGAGAGGAGAACCTTCCAGAAGAACAACCATCTCTGCAGCACTCCACCAATCAGGCCTGTATG

GTAGAGTGGCCAGACGGAAGCCACTCCTCAGTAAAAGGCACATGAAAGCCCACCTGGAGTTTGCCAA

AAGGCACGTGAAGGACTCTCAGACCAAAGATTGAACAAAGATTGATCTCTTTGGCCTGAATGGCAAGT

GTCATTTCTTGAAGAAAACCAGGCACAACTCATCACCTGGCCAATACCGTCCCTACCGTGAAGCATGG

LRS1_sgRNA PAM cut site

TGGTGGAAGCATCATGCTGTGGGGATGTTTTCAGTGGCAGGAACTGGGAGACTAGTCCACTCCTCAG

TAAAAGGCACATGAAAGCCCACCTGGAGTTTGCCAAAAGGCACGTGAAGGACTCTCAGACCAAAGAT

TGAACAAAGATTGATCTCTTTGGCCTGAATGGCAAGTGTCATTTCTTGAAGAAAACCAGGCACAACTC

left homology arm

ATCACCTGGCCAATACCGTCCCTACCGTGAAGCATGGTGGTGGAAGCATCATGCTACCAGCAAAGTTC

TAGAATTTGTCGAAACATTTATGTTATATATTTCCTGAAAAAAATTCTGAGTAAGTTCTTAAGTGTTAT

LRS1_E2_R

TGCCAGCAACATAAACAACAGACGGCAAAATGAATAAATGATAACAAAGCAGTAGGCTTAAATAAAC

CTAATTTTTATAGGCTGTTCTCTACAACCCTCAAACAGTGATTAGTTTTGTACTTATAAACTTGCCCTTT

CATTCATATTTCAAGAAAATTGGTTCAGAAGATCTGGATATTCTAGCAGTTGTTCAAGCTCATGGAGG

GATCAGTGACCTGATTCCAAATGACTAGGCCTAATCCAGAAATTAGATGACTGTCAACATAAAAAGGC

ACAGCACTCACTAGCTGCCCTATATATTTTATTATATTTTACATATATTATTTTATTTATTTAGCTCTGA

UBI promoter

GTGCTGTACTTTCTGGTTAAAGAAAACTGCTTACAACAGCTAACCTGTACTACCTCAGGCTCAGGGAA

TTTGGAACAGGTTTGTCTGGTTTGTTTCTTTAACCATGCATGCTTGTTTTCAACTATGGCAACACAGTCA

CATGGGACATTACAGAAATGATTTGTCGATGACATGCGACTTTTCTTTAATAAAGCGCAAAGATCCCA

AAAAGCAAACTTTTAACAAAAATCATATAATTATATTTTCAATCCAGCTTTGTAGCAACTTTGTGCTGC

TGTTCACTCAGCAACAGATAGTCAGTATAAGGTCAGTGTGTCTCAAAGCAGTGCCATCTGTTTCACAC

ATTGCGTTCTATATATAAGTGTGCTGGTTGACACGACACTGTATAAGGCCTAGGCTAAAACACAAACA

ATGTAGAATGACACTGTGTTTTTTTGTAAACAAATGTTGTTTTGGTTAAACATCTTTGTGAAAACAT

CCTCCTGTCATGTATTTGCTATATTCAAATGTTAAACCCGTGCAGAATAGAACATATACAAAAAAAAA

CAACACAACACATTTTTAAACATTATTAAATATCAAGTATTGCTGGCAGTTCTGTTTCTGTTTTACAGT

ACCCTTTGCCACAGTTCTCCGCTTTTCCTGGTCCAGATTCCACAAGTCTGATTCACCAATAGCAAAGCG

AATAAACAACCAAAGCAGCCAATCACTGCTTGTAGACTGTCCTGCGAGACCGGCCCATTCCAGCACAT

TCTGGAAACTTCCTTTATATGATAATTATAAATACATTTAAATTATTGATACAAAACATGTAATTCCTA

GAACATAACCATAGCAATCATTAGTTTCAGGGTAATTATGTATTTTTAGGATTTGACTGCGGAAAGAT

CTGGTCATGTGACGTCTCATGAACGTCACGGCCCTGGGTTTCTATAAATACAGTAGGACTCTCGACCAT

CGGCAGATTTTTCGAAGAAGAAGATCAGTTTCAGGAGCCGTACTGTTCCGTTATGAACCATTTACAAA

Elovl2 R

GTTTGGACGAGCGGTTAAACGCGCTTTTTACTTCCTGTTTGAAGACAGAGACTCCAGGGTACGAGGA

TGGCTCCTGTTGGACTCATATCTCCCCACCCTCTCCCTCACCATCCTTTACCTCCTCACAGTATACCTGG

GATCAAAATACATGAGGAACAGGCCAGCATACTCACTCAAGGGGGTCTTACAAGTGTACAACTTCTCT

GTGACCATGCTCTCCCTGTACATGCTGGTTGAGCTTGTCTTGGCAACCTTGTCGGCAGGCTACCGTCTG

E2_LRS1 _F

CAGTGTCAGGGACTTCACGAGGCAGGGGAGGCCGACCTCAGGGTAGCCAAGGTGCTGTGGTGGTACT

ACTTCTCCAAGGTGATTGAGTTCCTGGACACCATCTTCTTTGTGCTGCGGAAGAAGAACAGCCAGATC

ACCTTCCTGCACGTTTATCACCACGCCTCCATGTTCAACATTTGGTGGTGTGTTCTGAACTGGATCCCCT

GTGGACAGAGTTTCTTTGGGCCGACTCTTAACAGTTTCATCCATGTGTGTATGTACTCCTACTATGGCC

Masu salmon *elovl2*

TGTCCACTATACCCTCCATGCAGAAATACCTCTGGTGGAAGCGCTACCTGACTCAGGCTCAGTTGATCC

Elovl2 F

AGTTTATACTGACCATCACACACACCCTGTCTGCAATCGTCGTCCCTTGTGGTTTCCCTGTTGGATGCCT

GCTCTTCCAGTTCTCCTACATGGCCACCCTCGTCATCCTTTTTGTCAACTTCTACGTCCAGACCTACAGA

AAAAGACGGCCAGAAGAATCCATCAAGTCCAGTCGCCCAAATGGCCACTCTGTCTCCACCAATGGCAC

CAGCTTCAAGAAGAGAAAGTAGGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACA

ACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATT

ATAAGCTGCAATAAACAAGTTGTGGGGATGTTTTTCAGTGGCAGGAACTGGGAGACTAGTCAGGATCA

AGGGAAAGATGAATGCAGCAATGTAGAGACATCCTTGATGAAAACCTGCTCCAGAGCGCTCTGGACC

right homology arm

TCAGACTGGGGCGAAGGTTCATCTTCCAACAGGACAATGACCCTAAGCACACAGCCAAGATAACGAA

cut site   LRS1_sgRNA   PAM

GGAGAGGCTACAGGACAACCCGTCCCTACCGTGAAGCATGGTGGTGGAAGCATCATGCTGTGGGGAT

GTTTTTCAGTGGCAGGAACTGGGAGACTAGTCAGGATCAAGGGAAAGATGAATGCAGCAATGTAGAG

ACATCCTTGATGAAAACCTGCTCCAGAGCGCTCTGGACCTCAGACTGGGGCGAAGGTTCATCTTCCAA

LRS1

CAGGACAATGACCCTAAGCACACAGCCAAGATAACGAAGGAGAGGCTACAGGACAACTCTGTGAATG

E2_LRS1 _R

TCCTTGAGTGTCCCAGCCAGAGCCC

Figure S4. Sequence at LRS1 after insertion of the UBI_masu_salmon_*elovl2* donor plasmid with perfect NHEJ-mediated knock-in

CGCATTGTAAATCAGGTGTATTATCAATGTTCAGACTTTCAGCTGCTTGCGATGAACAAATCAAACAA

AATCAATTGAAATAGTTCGACACGACGAACGCTTCAAGCGGCTTCAACAAATTCAACTGAGAATGCAA

LRR2_D4_F

CTTATAATGACTTCTCCAGTCTCAAAATTATTCACCCCCCTGAATAGAATCCCGCCCAACAGCACAGAT

LRS2

ATGCAGAACAGGTGTTGACTCAAGCACACCTGATATAAACTAATCAAGAGCTTTATTAGTTGCAGCAG

GTGTGCTTGAGCTGGAACACATGAAATACCGGAACTGACTCGGGGCAGAAGATATATGAAAATCCTG

GAGGGGGCAGAAAAAGGAAGCTATCGACGGCTGCAGGCAGATTTGTGAGAAGGCAGGTTGTGAAAA

ACCCTCGAGTGACTGTAAAAGACCTGCAGCGAGACCTGGTGTAACAGGCGCTGAGGTTTCAGTGAGCG

CAGTAAGGCGCGTACTAAACTCAGTGTTTCCATGCCAGAAGTCCAAGACGTTCACCGCTACTGACCCA

AAAGCACAAGAAACGTCGCTCAGAATCATATAAATAATCCACAGAGGTTTGGGATTGTGTTCTGTGGA

left homology arm

GCGATGAGACTAAACTGGAACGTTGCAGCACGATGGATCAGCGGTACGTCTGGAGGAAGAAGAATGA

AGAAAGAACACTCTGTCCACAGTCGAGCATGGTGGAGGCTCGGTGATGCTACCAGCAAAGTTCTAGA

ATTTGTCGAAACATTTATGTTATATATTTCCTGAAAAAAATTCTGAGTAAGTTCTTAAGTGTTATTGCC

LRR2_D4_R

AGCAACATAAACAACAGACGGCAAAATGAATAAATGATAACAAAGCAGTAGGCTTAAATAAACCTAA

TTTTTATAGGCTGTTCTCTACAACCCTCAAACAGTGATTAGTTTTGTACTTATAAACTTGCCCTTTCATT

CATATTTCAAGAAAATTGGTTCAGAAGATCTGGATATTCTAGCAGTTGTTCAAGCTCATGGAGGGATC

AGTGACCTGATTCCAAATGACTAGGCCTAATCCAGAAATTAGATGACTGTCAACATAAAAAGGCACAG

CACTCACTAGCTGCCCTATATATTTTATTATATTTTACATATATTATTTTATTTATTTAGCTCTGAGTGCT

GTACTTTCTGGTTAAAGAAAACTGCTTACAACAGCTAACCTGTACTACCTCAGGCTCAGGGAATTTGG

AACAGGTTTGTCTGGTTTGTTTCTTTAACCATGCATGCTTGTTTTCAACTATGGCAACACAGTCACATG

GGACATTACAGAAATGATTTGTCGATGACATGCGACTTTTCTTTAATAAAGCGCAAAGATCCCAAAAA

GCAAACTTTTAACAAAAATCATATAATTATATTTTCAATCCAGCTTTGTAGCAACTTTGTGCTGCTGTT

UBI promoter

CACTCAGCAACAGATAGTCAGTATAAGGTCAGTGTGTCTCAAAGCAGTGCCATCTGTTTCACACATTG

CGTTCTATATATAAGTGTGCTGGTTGACACGACACTGTATAAGGCCTAGGCTAAAACACAAACAATGT

AGAATGACACTGTGTTTTTTTTGTAAACAAATGTTGTTTTTGGTTAAACATCTTTGTGAAAACATCCTCC

TGTCATGTATTTGCTATATTCAAATGTTAAACCCGTGCAGAATAGAACATATACAAAAAAAAACAACA

CAACACATTTTTAAACATTATTAAATATCAAGTATTGCTGGCAGTTCTGTTTCTGTTTTACAGTACCCTT

TGCCACAGTTCTCCGCTTTTCCTGGTCCAGATTCCACAAGTCTGATTCACCAATAGCAAAGCGAATAAA

CAACCAAAGCAGCCAATCACTGCTTGTAGACTGTCCTGCGAGACCGGCCCATTCCAGCACATTCTGGA

AACTTCCTTTATATGATAATTATAAATACATTTAAATTATTGATACAAAACATGTAATTCCTAGAACAT

AACCATAGCAATCATTAGTTTTCAGGGTAATTATGTATTTTTAGGATTTGACTGCGGAAAGATCTGGTC

ATGTGACGTCTCATGAACGTCACGGCCCTGGGTTTCTATAAATACAGTAGGACTCTCGACCATCGGCA

GATTTTTCGAAGAAGAAGATCAGTTTCAGGAGCCGTACTGTTCCGTTATGGGAGGTGGAGGTCAGCTG

GGGGAGTCAGGGGAGAATGGCTGCAAGTCAGCTGCTGGTGTGTACACTTGGGAGGAGGTGCAGCACC

Delta4_F

ACAGCAACAGGAATGACCAGTGGTTGGTCATTGATCGCAAGGTTTATAATGTCACCCAGTGGGCCAAG

AGACACCCAGGAGGGTTTCGGGTCCTCAACCACTATGCTGGAGAGGATGCTACGGAGGCATTCACTGC

TTTTCACCCCGACATAAAATTTGTACAAAAGTATATGAAGCCTTTGCTGGTAGGAGAGCTGGCTGCAA

CGGAGCCCGGTCAGGATCAAGACAAAAATGCCGCACTCATACAGGATTTCCACACTTTACGTCAGCAA

GCGGAGAGTGAGGGTCTGTTTCAAGCTCGCCCTTTGTTCTTCCTCCTTCATTTGGGTCACATCCTGTTGC

TGGAGGCTCTGGCCCTTCTGATGGTCTGGCACTGGGGAACGGGCTGGATACAGACGTTACTATGTGCC

GTTATGCTGGCAACTGCTCAGTCTCAGGCCGGCTGGCTTCAGCACGACTTTGGACACCTGTCTGTCTTC

AAGAAATCCCGCTGGAATCACTTGGTTCACAAGTTTGTCATTGGCCATTTAAAGGGAGCTTCTGCCAA

CTGGTGGAATCATCGTCATTTCCAGCATCACGCTAAACCCAACATCTTCAAGAAAGATCCTGACATCA

rabbitfish Δ4

ACATGGTGGACCTTTTTGTACTTGGAGAGACTCAACCTGTGGAGTACGGCATAAAGAAGATTAAAAAT

ATGCCCTATAACCACCAGCACAAGTATTTCTTTTTGGTTGCGCCACCACTTCTTATTCCAGTTTTCTACA

Delta4_R

ACTATAACATAATGATGACCATGATTACTCGCCGTGACTATGTGGATCTGTCTTGGGCCATGACGTTTT

ACATTCGCTACATGTTGTGCTATGTGCCGGTCTATGGCCTTTTTGGATCACTGGCGCTCATGATGTTTGC

CAGGTTTTTGGAGAGCCACTGGTTCGTGTGGGTAACTCAGATGAGTCATCTGCCCATGGACATCGACA

ATGACAAACGCCGTGACTGGCTGTCCATGCAGTTACAAGCCACCTGTAACATTGAGAAGTCTTTTTTCA

D4_LRR2_F

ACGACTGGTTCAGTGGACACCTCAACTTCCAAATCGAACACCATTTGTTCCCGAGGATGCCGCGCCAC

AACTACCACCTGGTGGCTCCACAGGTCCAGACACTGTGTGAGAAACATGGAATTCCATACGAAGTGAA

AACGCTGTGGAAAGGCATGGTTGACGTCGTCAGGGCACTGAAAAAATCAGGAGACCTCTGGCTTGAT

GCATATCTCCATAAATGAGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAG

poly A

AATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAG

CTGCAATAAACAAGTTCTGCGGCTGCTTTGCATCCTCTGGCGCTGGAGACCTGCAGCGTGTGGAAGGC

GAGATGGAGTCAGTGAAGCATCAGGAAATCCCAGGAGAAAACATCACGCCGTCTGTGAGGAAGCTGA

right homology arm

AGCTTGGGAGTCACTGGACCTTCAAACAGGACAGTGATCCCAAACATACCTTAAATTCCACCAAGGCT

TGGGTGCAAAAGTCCTGGAAGATTCTACAGGGGCATCACGGTCACCTGACTTGAACCCCATAGAAAAT

CTCCGGTGGGATTTGAAGAAGGCGGTCGCAGCACGAAACCCAAGAACATTACTGACCTGGAGGACGT

TGCTCATGAGGAACGGGAGGAGATTCCTCAGGAACGCTGCAGAAGCTCTGCATCTCGTCTACAGCAGG

LRS2

TCATAACAGCAAGAGGAGCTCTACTGAGTACTAGAGATGCTCGCCATGAAGGGGGTGAATAATTTAG

AGACAGGAGAAGTCATAAGTTGCATAAAGATTAATATTTTTTATTAATCAAAAGAAATTTCACAATGA

D4_LRR2_R

ATTTTTTTTAAAAATTCTAAATTTTACAATGAAAAGCAAAAAACTTGCTTTAAATATTGACATTGGGGA

AAAAATACTTTTTTTTACCTCCTATTTTTCTTCTCGTTATATTTTCTTAGTTTTACAA

Figure S5. Sequence at LRS2 after insertion of the UBI_rabbitfish_Δ4 donor plasmid with perfect HDR-mediated knock-in

CGCATTGTAAATCAGGTGTATTATCAATGTTCAGACTTTCAGCTGCTTGCGATGAACAAATCAAACAA

AATCAATTGAAATAGTTCGACACGACGAACGCTTCAAGCGGCTTCAACAAATTCAACTGAGAATGCAA

LRR2_D4_F

CTTATAATGACTTCTCCAGTCTCAAAATTATTCACCCCCCTGAATAGAATCCCGCCCAACAGCACAGAT

ATGCAGAACAGGTGTTGACTCAAGCACACCTGATATAAACTAATCAAGAGCTTTATTAGTTGCAGCAG

GTGTGCTTGAGCTGGAACACATGAAATACCGGAACTGACTCGGGGCAGAAGATATATGAAAATCCTG

LRS2

GAGGGGGCAGAAAAAGGAAGCTATCGACGGCTGCAGGCAGATTTGTGAGAAGGCAGGTTGTGAAAA

ACCCTCGAGTGACTGTAAAAGACCTGCAGCGAGACCTGGTGTAACAGGCGCTGAGGTTTCAGTGAGCG

CAGTAAGGCGCGTACTAAACTCAGTGTTTCCATGCCAGAAGTCCAAGACGTTCACCGCTACTGACCCA

AAAGCACAAGAAACGTCGCTCAGAATCATATAAATAATCCACAGAGGTTTGGGATTGTGTTCTGTGGA

GCGATGAGACTAAACTGGAACGTTGCAGCACGATGGATCAGCGGTACGTCTGGAGGAAGAAGAATGA

cut site LRS2_sgRNA PAM

AGAAAGAACACTCTGTCCACAGTCGAGCATGGTGGAGGCTCGGTGATGCTCTGCGGCTGCTTTGCATC

CTCTGGCGCTGGAGACCTGCAGACCGCTACTGACCCAAAAGCACAAGAAACGTCGCTCAGAATCATAT

AAATAATCCACAGAGGTTTGGGATTGTGTTCTGTGGAGCGATGAGACTAAACTGGAACGTTGCAGCAC

left homology arm

GATGGATCAGCGGTACGTCTGGAGGAAGAAGAATGAAGAAAGAACACTCTGTCCACAGTCGAGCATG

GTGGAGGCTCGGTGATGCTACCAGCAAAGTTCTAGAATTTGTCGAAACATTTATGTTATATATTTCCTG

LRR2_D4_R

AAAAAAATTCTGAGTAAGTTCTTAAGTGTTATTGCCAGCAACATAAACAACAGACGGCAAAATGAATA

AATGATAACAAAGCAGTAGGCTTAAATAAACCTAATTTTTATAGGCTGTTCTCTACAACCCTCAAACA

GTGATTAGTTTTGTACTTATAAACTTGCCCTTTCATTCATATTTCAAGAAAATTGGTTCAGAAGATCTG

GATATTCTAGCAGTTGTTCAAGCTCATGGAGGGATCAGTGACCTGATTCCAAATGACTAGGCCTAATC

CAGAAATTAGATGACTGTCAACATAAAAAGGCACAGCACTCACTAGCTGCCCTATATATTTTATTATA

TTTTACATATATTATTTTATTTATTTAGCTCTGAGTGCTGTACTTTCTGGTTAAAGAAAACTGCTTACAA

CAGCTAACCTGTACTACCTCAGGCTCAGGGAATTTGGAACAGGTTTGTCTGGTTTGTTTCTTTAACCAT

GCATGCTTGTTTTCAACTATGGCAACACAGTCACATGGGACATTACAGAAATGATTTGTCGATGACAT

GCGACTTTTCTTTAATAAAGCGCAAAGATCCCAAAAAGCAAACTTTTAACAAAAATCATATAATTATA

UBI promoter

TTTTCAATCCAGCTTTGTAGCAACTTTGTGCTGCTGTTCACTCAGCAACAGATAGTCAGTATAAGGTCA

GTGTGTCTCAAAGCAGTGCCATCTGTTTCACACATTGCGTTCTATATATAAGTGTGCTGGTTGACACGA

CACTGTATAAGGCCTAGGCTAAAACACAAACAATGTAGAATGACACTGTGTTTTTTTGTAAACAAAT

GTTGTTTTTGGTTAAACATCTTTGTGAAAACATCCTCCTGTCATGTATTTGCTATATTCAAATGTTAAAC

CCGTGCAGAATAGAACATATACAAAAAAAAACAACACAACACATTTTTAAACATTATTAAATATCAAG

TATTGCTGGCAGTTCTGTTTCTGTTTTACAGTACCCTTTGCCACAGTTCTCCGCTTTTCCTGGTCCAGAT

TCCACAAGTCTGATTCACCAATAGCAAAGCGAATAAACAACCAAAGCAGCCAATCACTGCTTGTAGAC

TGTCCTGCGAGACCGGCCCATTCCAGCACATTCTGGAAACTTCCTTTATATGATAATTATAAATACATT

TAAATTATTGATACAAAACATGTAATTCCTAGAACATAACCATAGCAATCATTAGTTTTCAGGGTAATT

ATGTATTTTTAGGATTTGACTGCGGAAAGATCTGGTCATGTGACGTCTCATGAACGTCACGGCCCTGGG

TTTCTATAAATACAGTAGGACTCTCGACCATCGGCAGATTTTTCGAAGAAGAAGATCAGTTTCAGGAG

CCGTACTGTTCCGTTATGGGAGGTGGAGGTCAGCTGGGGGAGTCAGGGGAGAATGGCTGCAAGTCAG

CTGCTGGTGTGTACACTTGGGAGGAGGTGCAGCACCACAGCAACAGGAATGACCAGTGGTTGGTCATT

Delta4_F
GATCGCAAGGTTTATAATGTCACCCAGTGGGCCAAGAGACACCCAGGAGGGTTTCGGGTCCTCAACCA

CTATGCTGGAGAGGATGCTACGGAGGCATTCACTGCTTTTCACCCCGACATAAAATTTGTACAAAAGT

ATATGAAGCCTTTGCTGGTAGGAGAGCTGGCTGCAACGGAGCCCGGTCAGGATCAAGACAAAAATGC

CGCACTCATACAGGATTTCCACACTTTACGTCAGCAAGCGGAGAGTGAGGGTCTGTTTCAAGCTCGCC

CTTTGTTCTTCCTCCTTCATTTGGGTCACATCCTGTTGCTGGAGGCTCTGGCCCTTCTGATGGTCTGGCA

CTGGGGAACGGGCTGGATACAGACGTTACTATGTGCCGTTATGCTGGCAACTGCTCAGTCTCAGGCCG

GCTGGCTTCAGCACGACTTTGGACACCTGTCTGTCTTCAAGAAATCCCGCTGGAATCACTTGGTTCACA

AGTTTGTCATTGGCCATTTAAAGGGAGCTTCTGCCAACTGGTGGAATCATCGTCATTTCCAGCATCACG

rabbitfish Δ4
CTAAACCCAACATCTTCAAGAAAGATCCTGACATCAACATGGTGGACCTTTTTGTACTTGGAGAGACT

CAACCTGTGGAGTACGGCATAAAGAAGATTAAAAATATGCCCTATAACCACCAGCACAAGTATTTCTT

TTTGGTTGCGCCACCACTTCTTATTCCAGTTTTCTACAACTATAACATAATGATGACCATGATTACTCGC

Delta4_R
CGTGACTATGTGGATCTGTCTTGGGCCATGACGTTTTACATTCGCTACATGTTGTGCTATGTGCCGGTC

TATGGCCTTTTTGGATCACTGGCGCTCATGATGTTTGCCAGGTTTTTGGAGAGCCACTGGTTCGTGTGG

GTAACTCAGATGAGTCATCTGCCCATGGACATCGACAATGACAAACGCCGTGACTGGCTGTCCATGCA

GTTACAAGCCACCTGTAACATTGAGAAGTCTTTTTTCAACGACTGGTTCAGTGGACACCTCAACTTCCA

D4_LRR2_F
AATCGAACACCATTTGTTCCCGAGGATGCCGCGCCACAACTACCACCTGGTGGCTCCACAGGTCCAGA

CACTGTGTGAGAAACATGGAATTCCATACGAAGTGAAAACGCTGTGGAAAGGCATGGTTGACGTCGTC

AGGGCACTGAAAAAATCAGGAGACCTCTGGCTTGATGCATATCTCCATAAATGAGATCCAGACATGAT

Poly A
AAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAA

TTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTCTGCGGCTGCTTTGCAT

CCTCTGGCGCTGGAGACCTGCAGCGTGTGGAAGGCGAGATGGAGTCAGTGAAGCATCAGGAAATCCC

right homology arm

AGGAGAAAACATCACGCCGTCTGTGAGGAAGCTGAAGCTTGGGAGTCACTGGACCTTCAAACAGGAC

cut site

AGTGATCCCAAACATACCTTAAATTCCACCAAGGCTTGGGTGCAAAAGTCTCTGTCCACAGTCGAGCA

LRS2_sgRNA PAM

TGGTGGAGGCTCGGTGATGCTCTGCGGCTGCTTTGCATCCTCTGGCGCTGGAGACCTGCAGCGTGTGG

AAGGCGAGATGGAGTCAGTGAAGCATCAGGAAATCCCAGGAGAAAACATCACGCCGTCTGTGAGGAA

GCTGAAGCTTGGGAGTCACTGGACCTTCAAACAGGACAGTGATCCCAAACATACCTTAAATTCCACCA

AGGCTTGGGTGCAAAAGTCCTGGAAGATTCTACAGGGGCATCACGGTCACCTGACTTGAACCCCATAG

AAAATCTCCGGTGGGATTTGAAGAAGGCGGTCGCAGCACGAAACCCAAGAACATTACTGACCTGGAG

GACGTTGCTCATGAGGAACGGGAGGAGATTCCTCAGGAACGCTGCAGAAGCTCTGCATCTCGTCTACA

LRS2

GCAGGTCATAACAGCAAGAGGAGCTCTACTGAGTACTAGAGATGCTCGCCATGAAGGGGGTGAATAA

TTTAGAGACAGGAGAAGTCATAAGTTGCATAAAGATTAATATTTTTTATTAATCAAAAGAAATTTCAC

D4_LRR2_R

AATGAATTTTTTTTAAAAATTCTAAATTTTACAATGAAAAGCAAAAAACTTGCTTTAAATATTGACATT

GGGGAAAAAATACTTTTTTTTACCTCCTATTTTTCTTCTCGTTATATTTTCTTAGTTTTACAA

Figure S6. Sequence at LRS2 after insertion of the UBI_rabbitfish_Δ4 donor plasmid with perfect NHEJ-mediated knock-in

ATACAGTATCTCACAAAAGTGAGTGCACCCCTCACATTTTTGTAAATATTTGATTAGATCTTTTCATGT

GACGTCACTGAAGAAATGACACTTTGCTACAATGTAAAGTAGTGAGTGTACAGCTTGTGTAACAGTGT

LRS3

AAATTTGCTGTCCCCTCAAAATAACTCAACACACAGCCATTAATGTCTAAACCGCTGGCAGCAAAAGT

GAGTACACCCCTAAGTGAAAATGTCCAAATTGGGCCCAAAGTGTCAGTATTTTGTGTGGCCACCATTA

LRR3_D6_F

TTTTCCAGCACTGCCTTAACCCTCTTGGGCATGGAGTTCACCAGAGCTTCACAGGTTGCCACTGGAGTC

CTCTTCCACTCCTCCATGACGACATCACGGAGCTGGTGGATGTTAGAGACCTTGTGCTCCTCCACCTTC

CGTTTGAGGATGCCCCACAGATGCTCAATAGGGTTTAGGTCTGGAGACATGCTTGGCCAGTCCATCAC

CTTCACCCTCAGCTTCTTTAGCAAGGCAAAGGTCGTCTTGGAGGTGTGTTTGGGGTCGTTATCATGCTG

left homology arm

GAATACTACATACTGATCATGCTCTGCTTCAGTATGGCACAGTACATGCTGGCATTCATGGTTCCCTCA

ATGAACTGTAGCTCCCCAGTGCCGGCAGCACTCATGCAGCCCCAGACCAGCAAAGTTCTAGAATTTGT

CGAAACATTTATGTTATATATTTCCTGAAAAAAATTCTGAGTAAGTTCTTAAGTGTTATTGCCAGCAAC

ATAAACAACAGACGGCAAAATGAATAAATGATAACAAAGCAGTAGGCTTAAATAAACCTAATTTTTA

TAGGCTGTTCTCTACAACCCTCAAACAGTGATTAGTTTTGTACTTATAAACTTGCCCTTTCATTCATATT

LRR3_D6_R

TCAAGAAAATTGGTTCAGAAGATCTGGATATTCTAGCAGTTGTTCAAGCTCATGGAGGGATCAGTGAC

CTGATTCCAAATGACTAGGCCTAATCCAGAAATTAGATGACTGTCAACATAAAAAGGCACAGCACTCA

CTAGCTGCCCTATATATTTTATTATATTTTACATATATTATTTTATTTATTTAGCTCTGAGTGCTGTACTT

UBI promoter

TCTGGTTAAAGAAAACTGCTTACAACAGCTAACCTGTACTACCTCAGGCTCAGGGAATTTGGAACAGG

TTTGTCTGGTTTGTTTCTTTAACCATGCATGCTTGTTTTCAACTATGGCAACACAGTCACATGGGACATT

ACAGAAATGATTTGTCGATGACATGCGACTTTTCTTTAATAAAGCGCAAAGATCCCAAAAAGCAAACT

TTTAACAAAAATCATATAATTATATTTTCAATCCAGCTTTGTAGCAACTTTGTGCTGCTGTTCACTCAGC

AACAGATAGTCAGTATAAGGTCAGTGTGTCTCAAAGCAGTGCCATCTGTTTCACACATTGCGTTCTATA

TATAAGTGTGCTGGTTGACACGACACTGTATAAGGCCTAGGCTAAAACACAAACAATGTAGAATGACA

CTGTGTTTTTTTTGTAAACAAATGTTGTTTTTGGTTAAACATCTTTGTGAAAACATCCTCCTGTCATGTA

TTTGCTATATTCAAATGTTAAACCCGTGCAGAATAGAACATATACAAAAAAAAACAACACAACACATT

TTTAAACATTATTAAATATCAAGTATTGCTGGCAGTTCTGTTTCTGTTTTACAGTACCCTTTGCCACAGT

TCTCCGCTTTTCCTGGTCCAGATTCCACAAGTCTGATTCACCAATAGCAAAGCGAATAAACAACCAAA

GCAGCCAATCACTGCTTGTAGACTGTCCTGCGAGACCGGCCCATTCCAGCACATTCTGGAAACTTCCTT

TATATGATAATTATAAATACATTTAAATTATTGATACAAAACATGTAATTCCTAGAACATAACCATAGC

AATCATTAGTTTTCAGGGTAATTATGTATTTTTAGGATTTGACTGCGGAAAGATCTGGTCATGTGACGT

CTCATGAACGTCACGGCCCTGGGTTTCTATAAATACAGTAGGACTCTCGACCATCGGCAGATTTTTCGA

AGAAGAAGATCAGTTTCAGGAGCCGTACTGTTCCGTTATGGGAGGTGGAGGTCAGCCGAGGGAGTCA

GGGGAGCCCGGCAGCAGTCCAGCTGTGTACACCTGGGAGGAAGTGCAGCACCACTCCTCCAGGAATG

ACCAGTGGTTGGTGATCGATCGAAAAGTTTATAACATCACTCAGTGGGCCAAACGGCACCCAGGAGG

Delta6_F

GTACCGGGTGATTGGCCATTATGCTGGGGAAGATGCTACGGAAGCATTCACTGCTTTCCACCCTGACTT

GAAATTTGTGCAAAAGTTCCTCAAGCCTTTGCTGATAGGAGAGCTGGCAGCCACAGAGCCCAGCCAGG

ACCGAAACAAAAATGCTGCGCTCATACAGGATTTCCACACTTTACGTCAGCAAGCGGAGAGTGAGGGT

CTGTTTCAAGCTCGCCCTTTGTTCTTCCTCCTTCATTTGGGCCACATCCTGTTGCTGGAGGCTCTGGCCC

TTCTGATGGTCTGGCACTGGGGAACGGGCTGGATACAGACGTTGCTATGTGCCGTTATGCTGGCAACT

GCTCAGTCTCAGGCCGGCTGGCTTCAGCACGACTTTGGACACCTGTCTGTCTTCAAGAAATCCCGCTGG

rabbitfish Δ6

AATCACTTGGTTCACCACTTTGTCATCGGCCATTTAAAGGGAGCTTCTGCCAACTGGTGGAATCATCGT

CATTTCCAGCATCACGCTAAACCCAACATCTTCAAGAAGGATCCTGACATCAACATGGTGAACCTTTTT

GTACTTGGAGAGACTCAACCTGTGGAGTATGGCGTAAAGAAGATCAAATTAATGCCCTACAACCACCA

D6_LRR3_F

GCACCAGTACTTCCATCTCATTGGACCACCGCTTCTCATTCCAGTTTTTTCCACTACCAGTTGCTGAAA

Delta6_R

ATCATGATTTCTCACCGCTACTGGCTGGATCTGGTGTGGTGCTTGTCCTTCTACCTTCGGTACATGTGCT

GCTATGTGCCGGTCTACGGCCTTTTTGGATCTGTGGTACTCATTGTATTTACAAGGTTTTTGGAGAGCC

ACTGGTTCGTGTGGGTGACGCAGATGAATCATCTGCCGATGGACATCAACTATGAGAACCACAACGAC

TGGCTGTCCATGCAGTTACAAGCCACCTGTAATGTTGAGCAGTCTCTCTTCAACGACTGGTTCAGTGGA

CATCTCAACTTTCAAATCGAACACCATTTGTTTCCCACCATGCCGCGCCACAACTACCACCTGGTGGTT

CCACGGGTCCGTGCACTCTGTGAGAAACATGAGATACCATACCAGGTGAAGACACTGCCGCGGGCCTT

CGCTGATATCATCAGGTCACTGAAAAACTCAGGGGAGCTCTGGCTTGATGCATATCTCCATAAATGAG

ATCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATG

poly A

CTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTACC

ATGACACTCCCACCACCATGCTTGACTGTAGGCAAGACACACTTGTCTTTGTACTCCTCACCTGGTTGC

right homology arm

CGCCACACACGCTTGACACCATCTGAACCAAATAAGTTTATCTTGGTCTCATCAGACCACAGGACATG

D6_LRR3_R

GTTCCAGTAATCCATGTCCTTAGTCTGCTTGTCTTCAGCAAACTGTTTGCGGGCTTTCTTGTGCATCATC

TTTAGAAGAGGCTTCCTTCTGGGACGACAGCCATGCAGACCAATTTGATGCAGTGTGCGGCGTATGGT

CTGAGCACTGACAGGCTGACCCCCCACCCCTTCAACCTCTGCAGCAATGCTGGCAGCACTCATACGTC

TATTTCCCAAAGACAACCTCTGGATATGACGCTGAGCACGTGCACTCAACTTCTTTGGTCGACCATGGC

GAGGCCTGTTCTGAGTGGAACCTGTCCTGTTAAACCGCTGTATGGTCTTGGCCACCGTGCTGCAGCTCA

GTGTCAGGGTCTTGGCAATCTTCTTATAGCCTAGGCCATCTTTATGTAGAGCAACAATTCTTTTTTTCAG

ATCCTCAGAGAGTTCTTTGCCATGAGGTGCCATGTTGAACTTCCAGTGACCAGTATGAGGGAGTGTGA

LRS3

GAGCGATGACACCAAATTTAACACACCTGCTCCCCATTCACACCTGAGACCTTGTAACACTAACAAGT

CACATGACACCGGGGAGGGAAAATGGCTAATTGGGCCCAATTTGGACATTTTCACTTAGGGGTGTACT

CACTTTTGCTGCCAGCGGTTTAGACATTAATGGCTGTGTGTTGAGTTATTTTGAGGGGACAGCAAATTT

ACACTGTTACACAAGCTGTACACTCACTACTTTACATTTTAGTAAAGTGTCATTTCTTCAGTGTTGTCAC

ATGAAAAGATCTAATCAAATATTTACAAAAATGTGAGGGGTGTACTCACTTTTGTGAGATACTGT

Figure S7. Sequence at LRS3 after insertion of the UBI_rabbitfish_Δ6 donor plasmid with perfect HDR-mediated knock-in

ATACAGTATCTCACAAAAGTGAGTGCACCCCTCACATTTTTGTAAATATTTGATTAGATCTTTTCATGT

GACGTCACTGAAGAAATGACACTTTGCTACAATGTAAAGTAGTGAGTGTACAGCTTGTGTAACAGTGT

LRS3

AAATTTGCTGTCCCCTCAAAATAACTCAACACACAGCCATTAATGTCTAAACCGCTGGCAGCAAAAGT

GAGTACACCCCTAAGTGAAAATGTCCAAATTGGGCCCAAAGTGTCAGTATTTTGTGTGGCCACCATTA

LRR3_D6_F

TTTTCCAGCACTGCCTTAACCCTCTTGGGCATGGAGTTCACCAGAGCTTCACAGGTTGCCACTGGAGTC

CTCTTCCACTCCTCCATGACGACATCACGGAGCTGGTGGATGTTAGAGACCTTGTGCTCCTCCACCTTC

CGTTTGAGGATGCCCCACAGATGCTCAATAGGGTTTAGGTCTGGAGACATGCTTGGCCAGTCCATCAC

CTTCACCCTCAGCTTCTTTAGCAAGGCAAAGGTCGTCTTGGAGGTGTGTTTGGGGTCGTTATCATGCTG

GAATACTACATACTGATCATGCTCTGCTTCAGTATGGCACAGTACATGCTGGCATTCATGGTTCCCTCA

cut site PAM LRS3_sgRNA

ATGAACTGTAGCTCCCCAGTGCCGGCAGCACTCATGCAGCCCCAGACCATGACACTCCCACCACCATG

CTTGACTGTAGGCAAGACTTGGCCAGTCCATCACCTTCACCCTCAGCTTCTTTAGCAAGGCAAAGGTCG

left homology arm

TCTTGGAGGTGTGTTTGGGGTCGTTATCATGCTGGAATACTACATACTGATCATGCTCTGCTTCAGTAT

GGCACAGTACATGCTGGCATTCATGGTTCCCTCAATGAACTGTAGCTCCCCAGTGCCGGCAGCACTCA

TGCAGCCCCAGACCAGCAAAGTTCTAGAATTTGTCGAAACATTTATGTTATATATTTCCTGAAAAAAT

TCTGAGTAAGTTCTTAAGTGTTATTGCCAGCAACATAAACAACAGACGGCAAAATGAATAAATGATAA

CAAAGCAGTAGGCTTAAATAAACCTAATTTTTATAGGCTGTTCTCTACAACCCTCAAACAGTGATTAGT

TTTGTACTTATAAACTTGCCCTTTCATTCATATTTCAAGAAAATTGGTTCAGAAGATCTGGATATTCTA

LRR3_D6_R

GCAGTTGTTCAAGCTCATGGAGGGATCAGTGACCTGATTCCAAATGACTAGGCCTAATCCAGAAATTA

GATGACTGTCAACATAAAAAGGCACAGCACTCACTAGCTGCCCTATATATTTTATTATATTTTACATAT

ATTATTTTATTTATTTAGCTCTGAGTGCTGTACTTTCTGGTTAAAGAAAACTGCTTACAACAGCTAACCT

GTACTACCTCAGGCTCAGGGAATTTGGAACAGGTTTGTCTGGTTTGTTTCTTTAACCATGCATGCTTGT

TTTCAACTATGGCAACACAGTCACATGGGACATTACAGAAATGATTTGTCGATGACATGCGACTTTTCT

TTAATAAAGCGCAAAGATCCCAAAAAGCAAACTTTTAACAAAAATCATATAATTATATTTTCAATCCA

GCTTTGTAGCAACTTTGTGCTGCTGTTCACTCAGCAACAGATAGTCAGTATAAGGTCAGTGTGTCTCAA

UBI promoter

AGCAGTGCCATCTGTTTCACACATTGCGTTCTATATATAAGTGTGCTGGTTGACACGACACTGTATAAG

GCCTAGGCTAAAACACAAACAATGTAGAATGACACTGTGTTTTTTTTGTAAACAAATGTTGTTTTTGGT

TAAACATCTTTGTGAAAACATCCTCCTGTCATGTATTTGCTATATTCAAATGTTAAACCCGTGCAGAAT

AGAACATATACAAAAAAAAACAACACAACACATTTTTAAACATTATTAAATATCAAGTATTGCTGGCA

GTTCTGTTTCTGTTTTACAGTACCCTTTGCCACAGTTCTCCGCTTTTCCTGGTCCAGATTCCACAAGTCT

GATTCACCAATAGCAAAGCGAATAAACAACCAAAGCAGCCAATCACTGCTTGTAGACTGTCCTGCGAG

ACCGGCCCATTCCAGCACATTCTGGAAACTTCCTTTATATGATAATTATAAATACATTTAAATTATTGA

TACAAAACATGTAATTCCTAGAACATAACCATAGCAATCATTAGTTTTCAGGGTAATTATGTATTTTTA

GGATTTGACTGCGGAAAGATCTGGTCATGTGACGTCTCATGAACGTCACGGCCCTGGGTTTCTATAAA

TACAGTAGGACTCTCGACCATCGGCAGATTTTTCGAAGAAGAAGATCAGTTTCAGGAGCCGTACTGTT

CCGTTATGGGAGGTGGAGGTCAGCCGAGGGAGTCAGGGGAGCCCGGCAGCAGTCCAGCTGTGTACAC

CTGGGAGGAAGTGCAGCACCACTCCTCCAGGAATGACCAGTGGTTGGTGATCGATCGAAAAGTTTATA

ACATCACTCAGTGGGCCAAACGGCACCCAGGAGGGTACCGGGTGATTGGCCATTATGCTGGGGAAGA

Delta6_F

TGCTACGGAAGCATTCACTGCTTTCCACCCTGACTTGAAATTTGTGCAAAAGTTCCTCAAGCCTTTGCT

GATAGGAGAGCTGGCAGCCACAGAGCCCAGCCAGGACCGAAACAAAAATGCTGCGCTCATACAGGAT

TTCCACACTTTACGTCAGCAAGCGGAGAGTGAGGGTCTGTTTCAAGCTCGCCCTTTGTTCTTCCTCCTT

CATTTGGGCCACATCCTGTTGCTGGAGGCTCTGGCCCTTCTGATGGTCTGGCACTGGGGAACGGGCTG

GATACAGACGTTGCTATGTGCCGTTATGCTGGCAACTGCTCAGTCTCAGGCCGGCTGGCTTCAGCACG

ACTTTGGACACCTGTCTGTCTTCAAGAAATCCCGCTGGAATCACTTGGTTCACCACTTTGTCATCGGCC

ATTTAAAGGGAGCTTCTGCCAACTGGTGGAATCATCGTCATTTCCAGCATCACGCTAAACCCAACATCT

rabbitfish Δ6

TCAAGAAGGATCCTGACATCAACATGGTGAACCTTTTTGTACTTGGAGAGACTCAACCTGTGGAGTAT

D6_LRR3_F

GGCGTAAAGAAGATCAAATTAATGCCCTACAACCACCAGCACCAGTACTTCCATCTCATTGGACCACC

GCTTCTCATTCCAGTTTTTTTCCACTACCAGTTGCTGAAAATCATGATTTCTCACCGCTACTGGCTGGAT

Delta6_R

CTGGTGTGGTGCTTGTCCTTCTACCTTCGGTACATGTGCTGCTATGTGCCGGTCTACGGCCTTTTTGGAT

CTGTGGTACTCATTGTATTTACAAGGTTTTTGGAGAGCCACTGGTTCGTGTGGGTGACGCAGATGAATC

ATCTGCCGATGGACATCAACTATGAGAACCACAACGACTGGCTGTCCATGCAGTTACAAGCCACCTGT

AATGTTGAGCAGTCTCTCTTCAACGACTGGTTCAGTGGACATCTCAACTTTCAAATCGAACACCATTTG

TTTCCCACCATGCCGCGCCACAACTACCACCTGGTGGTTCCACGGGTCCGTGCACTCTGTGAGAAACAT

GAGATACCATACCAGGTGAAGACACTGCCGCGGGCCTTCGCTGATATCATCAGGTCACTGAAAAACTC

AGGGGAGCTCTGGCTTGATGCATATCTCCATAAATGAGATCCAGACATGATAAGATACATTGATGAGT

poly A

TTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTT

TATTTGTAACCATTATAAGCTGCAATAAACAAGTTACCATGACACTCCCACCACCATGCTTGACTGTAG

GCAAGACACACTTGTCTTTGTACTCCTCACCTGGTTGCCGCCACACACGCTTGACACCATCTGAACCAA
right homology arm
ATAAGTTTATCTTGGTCTCATCAGACCACAGGACATGGTTCCAGTAATCCATGTCCTTAGTCTGCTTGT
cut site
PAM
CTTCAGCAAACTGTTTGCGGGCTTTCTTCTGTAGCTCCCCAGTGCCGGCAGCACTCATGCAGCCCCAGA
LRS3_sgRNA
CCATGACACTCCCACCACCATGCTTGACTGTAGGCAAGACACACTTGTCTTTGTACTCCTCACCTGGTT

GCCGCCACACACGCTTGACACCATCTGAACCAAATAAGTTTATCTTGGTCTCATCAGACCACAGGACA

D6_LRR3_R
TGGTTCCAGTAATCCATGTCCTTAGTCTGCTTGTCTTCAGCAAACTGTTTGCGGGCTTTCTTGTGCATCA

TCTTTAGAAGAGGCTTCCTTCTGGGACGACAGCCATGCAGACCAATTTGATGCAGTGTGCGGCGTATG

GTCTGAGCACTGACAGGCTGACCCCCCACCCCTTCAACCTCTGCAGCAATGCTGGCAGCACTCATACG

TCTATTTCCCAAAGACAACCTCTGGATATGACGCTGAGCACGTGCACTCAACTTCTTTGGTCGACCATG

GCGAGGCCTGTTCTGAGTGGAACCTGTCCTGTTAAACCGCTGTATGGTCTTGGCCACCGTGCTGCAGCT

LRS3
CAGTGTCAGGGTCTTGGCAATCTTCTTATAGCCTAGGCCATCTTTATGTAGAGCAACAATTCTTTTTTC

AGATCCTCAGAGAGTTCTTTGCCATGAGGTGCCATGTTGAACTTCCAGTGACCAGTATGAGGGAGTGT

GAGAGCGATGACACCAAATTTAACACACCTGCTCCCCATTCACACCTGAGACCTTGTAACACTAACAA

GTCACATGACACCGGGGAGGGAAAATGGCTAATTGGGCCCAATTTGGACATTTTCACTTAGGGGTGTA

CTCACTTTTGCTGCCAGCGGTTTAGACATTAATGGCTGTGTGTTGAGTTATTTTGAGGGGACAGCAAAT

TTACACTGTTACACAAGCTGTACACTCACTACTTTACATTTTAGTAAAGTGTCATTTCTTCAGTGTTGTC

ACATGAAAAGATCTAATCAAATATTTACAAAAATGTGAGGGGTGTACTCACTTTTGTGAGATACTGT

Figure S8. Sequence at LRS3 after insertion of the UBI_rabbitfish_Δ6 donor plasmid with perfect NHEJ-mediated knock-in