

Original Research Article

Molecular Cloning, Gene Expression in Albino Mutants and Gene Knockdown Studies of Tyrosinase mRNA in Rainbow Trout

SURINTORN BOONANUNTANASARN¹, GORO YOSHIZAKI², KENICHI IWAI² and TOSHIO TAKEUCHI²

¹School of Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, University Avenue, Muang, Nakhon Ratchasima, Thailand; ²Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Konan, Minato, Tokyo, Japan

*Address reprint requests to Dr Goro Yoshizaki, Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Konan, Minato, Tokyo 108-8477, Japan. E-mail: goro@s.kaiyodai.ac.jp

Received 22 January 2004; in final form 28 April 2004

Tyrosinase has a role in melanin synthesis and several defects of the tyrosinase gene lead to albinism. Here, we cloned and characterized rainbow trout tyrosinase cDNAs and carried out the molecular and biochemical characterization of albino mutants. Two types of cDNA were cloned: tyrosinase-1 (*Tyr-1*) and tyrosinase-2 (*Tyr-2*). Both contained regions predicted to encode structural features of tyrosinase, and phylogenetic analysis confirmed that *Tyr-1* and *Tyr-2* were members of the tyrosinase family. *Tyr-1* transcripts were first detected in embryos at 5 d post-fertilization (dpf) and *Tyr-2* transcripts at 15 dpf. 3,4-dihydroxyphenylalanine assays revealed significantly reduced tyrosinase activities in dominant and recessive albino mutants compared with wild-type embryos. However, reverse-transcription PCR showed no differences in the amounts or

lengths of the coding regions of *Tyr-1* and *Tyr-2* transcripts between wild-type embryos and albino mutants. Antisense morpholino oligonucleotides (AMOs) designed to knockdown tyrosinase gene expression in wild-type embryos led to reduced pigmentation in the retina and skin of embryos at 25 and 35 dpf, respectively. Furthermore, the tyrosinase activities of AMO-treated embryos were significantly reduced. We conclude that both *Tyr-1* and *Tyr-2* are crucial for melanin synthesis in rainbow trout embryos. Furthermore, we describe a potential application of AMOs in the treatment of hyperpigmentation.

Key words: Antisense morpholino oligonucleotides, Dominant albino mutant, Melanin, *Oncorhynchus mykiss*, Recessive albino mutant, Tyrosinase

INTRODUCTION

Tyrosinase (EC 1.14.18.1), which is also known as monophenol oxygenase, is a copper-containing glycoprotein that is localized in eyes, skin and hair (1). It is a key enzyme for melanin synthesis and catalyses the first two steps of this process: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopa-quinone (1, 2). Several defects of the tyrosinase gene can lead to albinism; for example, a point mutation in the tyrosinase gene is responsible for an albino phenotype in mice (3) and the insertion of a transposable element into this gene causes an albino phenotype in the medaka (4).

Among the salmonids, albino mutants have been reported in the amago salmon *Oncorhynchus masou ishikawai* (5), the brook trout *Salvelinus fontinalis* (6), the lake trout *Salvelinus namaycush* (7), the Chinook salmon *Oncorhynchus tshawytscha* (8) and the rainbow trout *Oncorhynchus mykiss*. In most cases, the albino phenotype is recessively inherited. Although the recessive albino-mutant rainbow trout has red eyes and white-yellow skin, a dominant albino-mutant strain was also reported at the Nagano Prefectural Fisheries Experimental Station (Nagano, Japan) in 1956 (9), which has black eyes and pale-orange skin. Despite the various reports of albino

Abbreviations – aa, amino acid; AMO, antisense morpholino oligonucleotides; bp, base pairs; DOPA, 3,4-dihydroxyphenylalanine; dpf, days post-fertilization; MO, morpholino oligonucleotides; nt, nucleotide; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcription PCR

salmonids, molecular and biochemical studies have not been carried out on these mutant strains.

The introduction of a functional tyrosinase gene into a tyrosinase-deficient albino-mutant host allows expression of the foreign gene to be easily detected through changes in body color. The transgenic rescue of an albino phenotype by the introduction of wild-type tyrosinase cDNA or the tyrosinase gene has been reported in both the mouse and the medaka (10, 11). The tyrosinase gene is therefore a useful reporter gene for transgenic studies and could help to improve transgenic techniques in commercially valuable species.

In addition, the potential use of albino fish in techniques such as blastomere and germ-cell transplantation (12, 13) has recently been recognized. Furthermore, the tyrosinase gene could have a role in the development of novel gene-knockout and gene-knockdown techniques, which are important systems in which to study the role of new genes.

However, despite its many potential applications, the tyrosinase gene has not been cloned from commercially valuable fish species such as the rainbow trout – a commercial species worldwide, which has been used as a model for the development of both transgenic and chimeric strains (14). The relationship between tyrosinase gene-expression and albinism in this species is still unclear. Therefore, as a first step towards the development of biotechnological systems utilizing albino rainbow trout, we cloned and characterized rainbow trout tyrosinase cDNAs. In addition, the molecular and biochemical characterization of albino mutants was carried out.

MATERIALS AND METHODS

Rainbow trout strains – wild-type (Fig. 1A) dominant albino (Fig. 1B) and recessive albino (Fig. 1C) – were reared for several generations at the Oizumi Research Station, Tokyo University of Marine Science and Technology (Yamanashi, Japan). Dominant and recessive albino mutants were

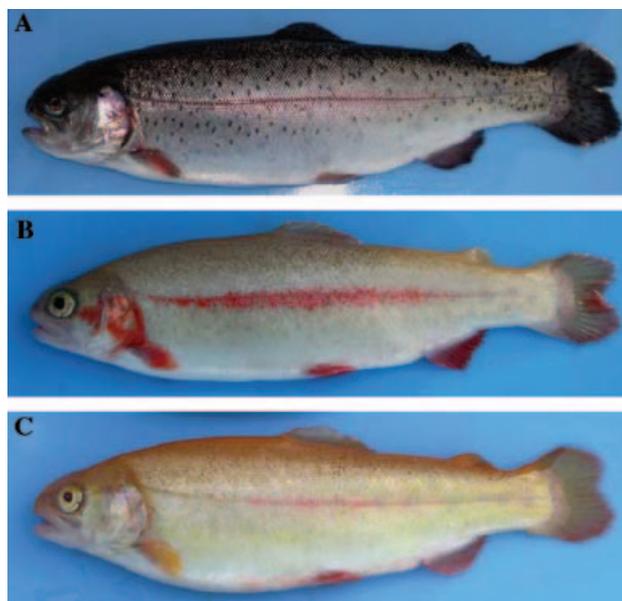


Fig. 1. Body color mutants of rainbow trout. Wild-type (A), dominant albino (B) and recessive albino (C).

provided by the Nagano and Tochigi Prefectural Fisheries Experimental Stations (Japan), respectively.

Molecular Cloning of Wild-type Tyrosinase cDNAs

To obtain a partial tyrosinase gene fragment, PCR was performed using genomic DNA extracted from the liver as a template, with a pair of primers (mTYR-F and mTYR-R; Table 1) that were designed using two highly conserved regions of the tyrosinase gene. PCR was carried out in a total volume of 10 μ l, consisting of 50 ng genomic DNA, 200 μ M of each deoxynucleotide, 1 pmol of each primer, 1X EX Taq

Table 1. Primers used for RACE-PCR and RT-PCR

Primer	Sequence	PCR	Primer order
mTYR-F	5'-AAGGAATGTTGTCCAGTCTGG-3'	Degenerate	Sense
mTYR-R	5'-GCATCTCTCCAGTCCCAGTATGG-3'	Degenerate	Antisense
5'A-SP1	5'-CAAACAGTCCGAAGTCC-3'	5' RACE	Antisense
5'A-SP2	5'-CGCAGTTGAACCCCATGTAGTTGGA-3'	5' RACE	Antisense (nested)
5'B-SP1	5'-TGC GCGGTTCTGCCATGGCACCGA-3'	5' RACE	Antisense
5'B-SP2	5'-TACCCACACACTGGGATAGACGACC-3'	5' RACE	Antisense (nested)
3'A-SP1	5'-ATGGCAACTCGGGGAGAGTTTCAGCAT-3'	3' RACE	Sense
3'A-SP2	5'-ACCGCTCATCATCAGCAGTGATAGTGATA-3'	3' RACE	Sense (nested)
3'B-SP1	5'-TCAGGACGTGACGGTATCGGAAGA-3'	3' RACE	Sense
3'B-SP2	5'-TGGGACCAGGAAATGTTTGCCCGACATA-3'	3' RACE	Sense (nested)
TyrA-F1	5'-TCTGTGTGACGGTAACGACG-3'	PCR	Sense
TyrA-R1	5'-CTGGACTGAAGACATTGAGC-3'	PCR	Antisense
TyrA-F2	5'-AGTTCACGGTCCAGTCTCCGT-3'	RT-PCR	Sense
TyrB-R1	5'-TCCCCCGTCACTCTCTGATCTCGT-3'	PCR	Antisense
TyrA-F3	5'-TGC GATGCCCTCCTTCTCATCCAC-3'	RT-PCR	Sense
TyrA-R3	5'-TAAATAGTGTGGTAGTTGGGGGTAT-3'	RT-PCR	Antisense
TyrA-F4	5'-TCCTCCAGTGTTCGCGTTCTCCCA-3'	RT-PCR	Sense (nested)
TyrA-R4	5'-TCACTGCTGATGATAAGCGGTTCCC-3'	RT-PCR	Antisense (nested)
TyrB-F2	5'-TGGTCAGTCATGGTCTTCTGGGTG-3'	RT-PCR	Sense
TyrB-R2	5'-TCTTCCTGTTCTGCTCCGCCTCCTG-3'	RT-PCR	Antisense
TyrB-F3	5'-TGGGTAGCTGCTCCAGATGCTGTT-3'	RT-PCR	Sense (nested)
TyrB-R3	5'-ACAGGGCCCCGGTCTGCATGACAAT-3'	RT-PCR	Antisense (nested)
β -actin-F	5'-ACTACCTCATGAAGATCCTG-3'	RT-PCR	Sense
β -actin-R	5'-TTGCTGATCCACATCTGCTG-3'	RT-PCR	Antisense

buffer and 0.25 U EX Taq (Takara Shuzo, Shiga, Japan). The PCR reaction was performed at 94°C for 3 min, then 30 reaction cycles were run – each consisting of 30 s at 94°C, 45 s at 55°C and 1 min at 72°C. The final-elongation step was conducted at 72°C for 5 min. DNA fragments of the predicted molecular weight, which were around 600 base pairs (bp) in length, were isolated using the Gelpure DNA Purification Kit (GeneMate, Kaysville, UT, USA). The PCR-amplified DNA fragments were cloned into the pGEM T-Easy plasmid (Promega, Madison, WI, USA) and sequenced. Two independent clones that potentially encoded tyrosinase were identified and designated as tyrosinase-1 (*Tyr-1*) and tyrosinase-2 (*Tyr-2*).

For full-length cloning of the cDNAs, RNA was isolated from the heads of newly hatched rainbow trout embryos using the TRIZOL Reagent (Gibco BRL, Rockville, MD, USA) according to the manufacturer's protocol. 5' rapid amplification of cDNA ends (RACE) was performed using the 5' RACE System Version 2.0 (Gibco BRL) according to the manufacturer's protocol. Two specific primers were designed for both *Tyr-1* (5'A-SP1 and 5'A-SP2) and *Tyr-2* (5'B-SP1 and 5'B-SP2) as shown in Table 1. The PCR product was purified, cloned into the pGEM T-Easy vector and used for DNA sequencing.

3' RACE was carried out using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Two specific primers were designed for both *Tyr-1* (3'A-SP1 and 3'A-SP2) and *Tyr-2* (3'B-SP1 and 3'B-SP2) as shown in Table 1. The PCR product was purified, cloned into the pGEM T-Easy vector and used for DNA sequencing.

DNA Sequencing

DNA purification was performed using the FlexiPrep Kit (Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK). Both strands were sequenced using the ALFexpress DNA Sequencer System (Amersham Pharmacia Biotech Ltd) with a Thermo Sequenase fluorescent labeled-primer cycle-sequencing kit and 7-deaza-dGTP (Amersham Pharmacia Biotech Ltd). A phylogenetic tree was generated using the neighbor-joining method (15).

In order to investigate the localization of *Tyr-1* and *Tyr-2* in different loci on the wild-type rainbow trout genome, we used homozygous gynogenetic embryos. The homozygous embryo was obtained by mitotic gynogenesis (16). Using genomic DNA as the template, the PCR was performed at 94°C for 3 min, then 40 reaction cycles were run, each consisting of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C. The final-elongation step was conducted at 72°C for 15 min. Two pairs of primers were designed to amplify DNA fragment of *Tyr-1* gene (TyrA-F1 and TyrA-R1) and *Tyr-2* gene (3'B-SP1 and TyrB-R1) (Table 1). The PCR products were cloned to pGEM T-Easy vector and analyzed by DNA sequencing.

RT-PCR with Early Embryos

The onset of tyrosinase gene expression during embryogenesis was investigated using RT-PCR. Total RNA was extracted from wild-type embryos that were dissected from the yolk sac

at various developmental stages. Approximately 2 µg total RNA was used for the first-strand cDNA synthesis. Reverse transcription was performed using Ready-To-Go You – Prime First Strand Beads (Amersham Pharmacia Biotech Ltd) with an oligo (dT) primer. PCR reactions were performed at 94°C for 3 min, then 40 reaction cycles were run – each consisting of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C. The final-elongation step was conducted at 72°C for 5 min. Table 1 shows the sequences of the specific primers used for *Tyr-1* (TyrA-F2 and TyrA-R1), *Tyr-2* (3'B-SP1 and TyrB-R1) and β-actin (β-actin-F and β-actin-R).

RT-PCR with Albino Mutant cDNAs

To examine the transcription of *Tyr-1* and *Tyr-2* in wild-type, dominant albino and recessive albino embryos, semi-quantitative reverse-transcription PCR (RT-PCR) was performed to measure the amount of each transcript. In addition, RT-PCR of the coding region was carried out to determine the length of the transcripts. Total RNA was extracted from the pooled head samples of hatched embryos using the TRIZOL Reagent (Gibco BRL) according to the manufacturer's protocol. Poly(A)⁺ RNA was purified using Oligotex-dT30 (Takara Shuzo) according to the manufacturer's protocol. Approximately 2.5 µg poly(A)⁺ RNA was used for the first-strand cDNA synthesis. Reverse Transcription was performed using Ready-To-Go You-Prime First Strand Beads (Amersham Pharmacia Biotech Ltd) with an oligo (dT) primer. For semi-quantitative PCR, the reaction was performed at 94°C for 3 min, then 30 reaction cycles were run, each consisting of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C, using the primers TyrA-F2 and TyrA-R1 or 3'B-SP1 and TyrB-R1 for *Tyr-1* or *Tyr-2*, respectively (Table 1). For amplification of the entire coding region, a PCR reaction was carried out at 94°C for 3 min, then 40 reaction cycles were run, each consisting of 45 s at 94°C, 45 s at 60°C and 3 min at 72°C. The final-elongation step was conducted at 72°C for 15 min. Four pairs of primers were designed to amplify the coding DNA regions of *Tyr-1* (TyrA-F3 and TyrA-R3) and *Tyr-2* (TyrB-F2 and TyrB-R2), and the nested primers of *Tyr-1* (TyrA-F4 and TyrA-R4) and *Tyr-2* (TyrB-F3 and TyrB-R3) (Table 1). The PCR products were purified, cloned into pGEM T-Easy vector and subsequently analyzed by DNA sequencing.

Tyrosinase-Activity Assay

To compare tyrosinase activity between wild-type, dominant albino and recessive albino embryos, a DOPA assay was carried out using pooled head samples from 100 hatched embryos per group. The soluble membrane-bound protein fraction containing the tyrosinase protein was isolated using a modification of the method described by Sato et al. (17). Briefly, the samples from each group were homogenized in twice their own volume of 0.5 M sucrose solution. The homogenate was centrifuged at 800 rpm at 4°C for 5 min, and the supernatant was then ultracentrifuged at 45 000 rpm (100 000 g) at 4°C for 1.5 h. The pellet was resuspended in a solution containing 0.5 M sucrose and 1% Triton-X, and was left on ice for 30 min. The resulting suspension was

ultracentrifuged, under the conditions described above, to produce a supernatant comprising the soluble membrane-bound protein fraction.

The tyrosinase activities of the isolated protein samples were evaluated using a colorimetric method, based on that described by Miyazaki and Seiji (18). Briefly, 150 µg of the soluble membrane-bound protein was diluted with PBS(-) to a final volume of 450 µl. The colorimetric reaction was initiated by adding 50 µl of 10 mM L-DOPA (Sigma, St Louis, MO, USA), then incubated at 20°C for 1.5 h, and finally photo-absorbance analysis was performed at λ 420 nm. To quantify the enzyme activity, mushroom tyrosinase (Sigma) was used as the standard.

Gene-Knockdown Experiment

Morpholino oligonucleotides (MOs) were purchased from Gene Tools, LLC (Philomath, OR, USA). Antisense MOs (AMOs) were designed against nucleotides -22 to +3 of *Tyr-1* mRNA (At*Tyr-1*; 5'-CATCGCAATACATCACACCTG

TCCC-3') and -8 to +17 of *Tyr-2* mRNA (At*Tyr-2*; 5'-ACACCCAGAAGAACCATGACTGATC-3') (sequences complementary to the predicted start codon are underlined). A random standard control oligonucleotide (Sc-MO) (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a negative control. For microinjection, each MO was dissolved in 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, and stored at -20°C until use.

Wild-type fish were used for studying gene knockdown. Milt was collected from 1-yr-old males and ripe eggs were harvested from 2-yr-old females. After fertilization, the eggs were activated and incubated in 1 mM glutathione (reduced) solution (pH 8.0) to prevent hardening of the chorion (19). Next, 2 nl of each solution, containing 5 ng of MO, was microinjected into the blastodisc of 1-cell-stage embryos 2-7 h after fertilization. To investigate whether the MOs interfered with gene expression, tyrosinase activity was measured in samples from the heads of embryos at 25 dpf, and from the heads and bodies of 35-dpf embryos, which were injected with each of the MOs.

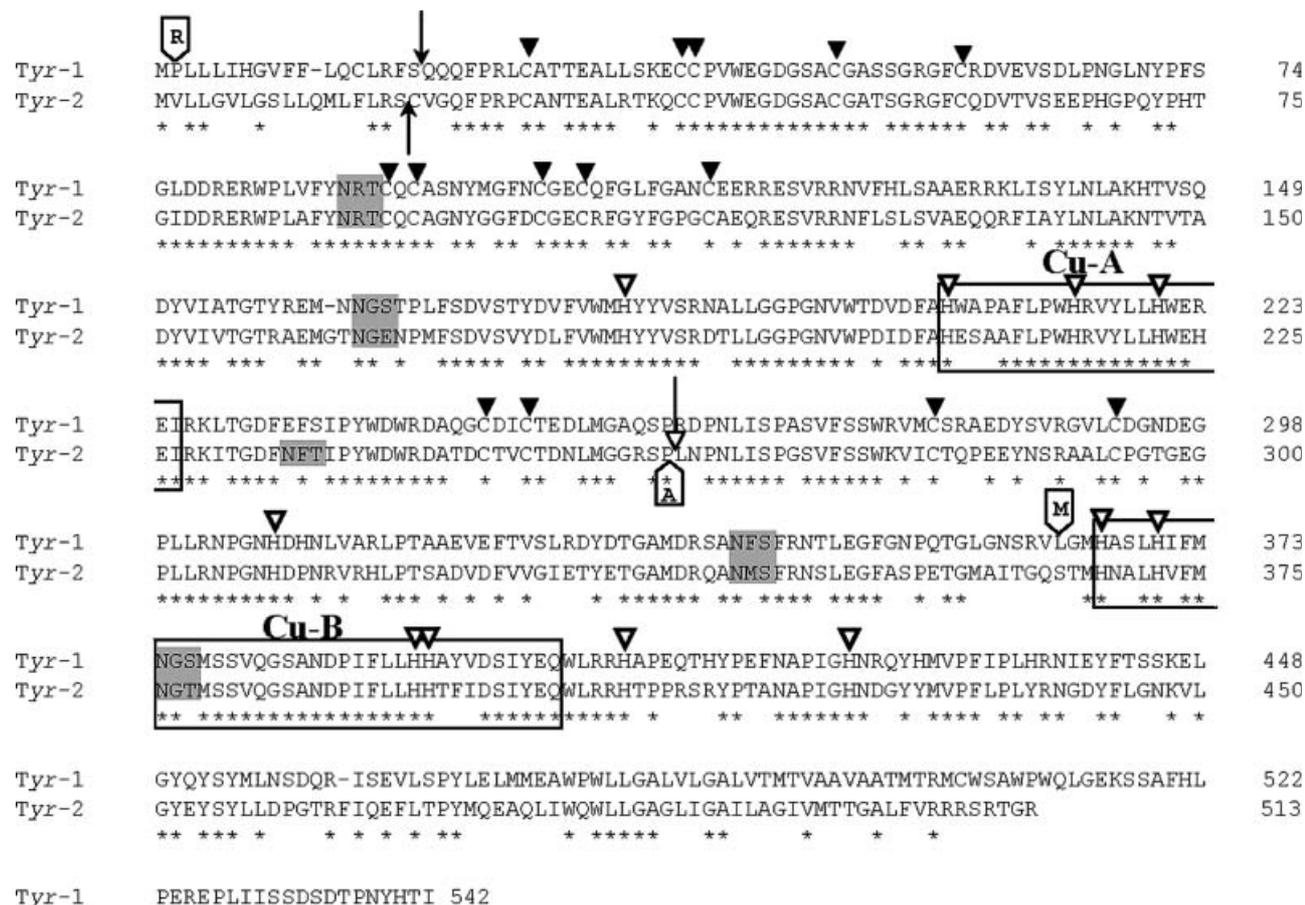


Fig. 2. Predicted aa sequences of wild-type *Tyr-1* and *Tyr-2*. The aa sequences were aligned using the Genetyx-WIN Version 3.1.0 software. Arrows indicate the cleavage site between the signal peptide and the mature protein. Homologies are indicated by asterisks below the sequences. Gaps introduced to maximize sequence homology are represented by a dash. Cysteine and histidine residues that are conserved between both tyrosinase genes are indicated by black and white arrow heads, respectively. Potential N-glycosylation sites are indicated by shaded boxes. The white boxes show the copper-A-binding site (Cu-A) and the copper-B-binding site (Cu-B). Amino acid substitutions of recessive albino *Tyr-1* and *Tyr-2* are indicated by pentagon boxes. Open arrow indicates the termination signal of the truncated recessive albino *Tyr-2*.

RESULTS

Cloning and Characterization of Tyrosinase cDNAs from Wild-type Rainbow Trout

DNA fragments of approximately 630 bp in length containing a motif that was predicted to encode the tyrosinase copper-A-binding region (20) were amplified by degenerate PCR using genomic DNA of wild-type rainbow trout as the template (data not shown). Sequencing these fragments revealed two different types, which we designated as *Tyr-1* and *Tyr-2*. The full-length cDNAs were also cloned.

The first cloned cDNA, *Tyr-1* (GenBank accession number AB117622), consisted of an open reading frame that encoded 542 amino acid (aa) residues (Fig. 2), approximately 180 bp of 5' untranslated region and 1.2 kb of 3' untranslated region. The predicted signal peptide for secretory or membrane-associated proteins (21) comprised the first 18 aa residues. The predicted molecular weight and theoretical isoelectric point of *Tyr-1* were 59.5 kDa and pH 5.42, respectively. A hydrophobic region that corresponded to the transmembrane domain was predicted at aa 476–502 (21). Four potential *N*-glycosylation sites (22) were predicted at aa positions 88–90, 163–165, 340–342 and 374–376 (as shown by the shaded boxes in Fig. 2). Two copper-binding sites, the copper-A-binding site and copper-B-binding site (20), were predicted at aa positions 205–225 and 366–402, respectively. In addition, starting from the N-terminus, four distinct domains were predicted: a cysteine-rich domain (domain I), a histidine-rich domain (domain II), a cysteine-rich domain (domain III) and a histidine-rich domain (domain IV) (Fig. 3).

The second cloned cDNA, *Tyr-2* (GenBank accession no. AB122031), had an open reading frame encoding 513 aa residues (Fig. 2), approximately 208 bp of 5' untranslated region and 1.4 kb of 3' untranslated region. Similar to *Tyr-1*, the signal peptide was predicted to span the first 18 aa residues. Therefore, the gene product was predicted to comprise 495 aa with a molecular weight of 55.6 kDa and an isoelectric point at pH 5.73. A hydrophobic region was predicted at the position of the transmembrane domain (aa 476–505) (21). Five potential *N*-glycosylation sites (22) were predicted at positions 89–91, 165–167, 235–237, 342–344 and 376–378 (as indicated by the shaded boxes in Fig. 2). Two copper-binding sites, the copper-A-binding site and the copper-B-binding site (20), were predicted at aa positions 207–227 and 368–404, respectively. In addition, *Tyr-2* was also predicted to contain the four distinct domains described in *Tyr-1* (Fig. 3).

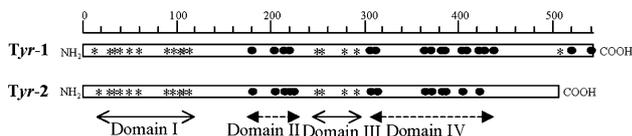


Fig. 3. Location of essential aa residues for catalytic activities in rainbow trout *Tyr-1* and *Tyr-2*. The four distinct cysteine- and histidine-rich domains (domains I–IV) reported by Kwon et al. (20) in human tyrosinase were also seen in rainbow trout *Tyr-1* and *Tyr-2*. Asterisks and circles indicate the position of the cysteine and histidine residues, respectively.

Further, DNA fragments of approximately 700 and 500 bp in lengths were amplified using genomic DNA template, that was prepared from a homozygous gynogenetic embryo and primer sets specific to *Tyr-1* and *Tyr-2*, respectively (data not shown). DNA sequencing analysis revealed that these two amplicons encoded *Tyr-1* and *Tyr-2* (data not shown). *Tyr-1* and *Tyr-2* had high identity with other known vertebrate tyrosinase cDNAs, as shown in Table 2. Phylogenetic analysis indicated that *Tyr-1* and *Tyr-2* were closely related to the teleost tyrosinases (Fig. 4).

Table 2. Percentage identity values of amino acids sequenced between trout and other vertebrate tyrosinases

Species (GenBank accession no.)	Tyrosinase-1	Tyrosinase-2
Human (M27160)	59.2	59.3
Mouse (D00440)	59.4	61.3
Chicken (D88349)	57.9	59.8
Frog (D12514)	58.1	58.5
Zebrafish (AF542067)	65.0	72.4
Medaka (D29687)	60.7	77.8
Fugu (AF343911)	61.8	74.9

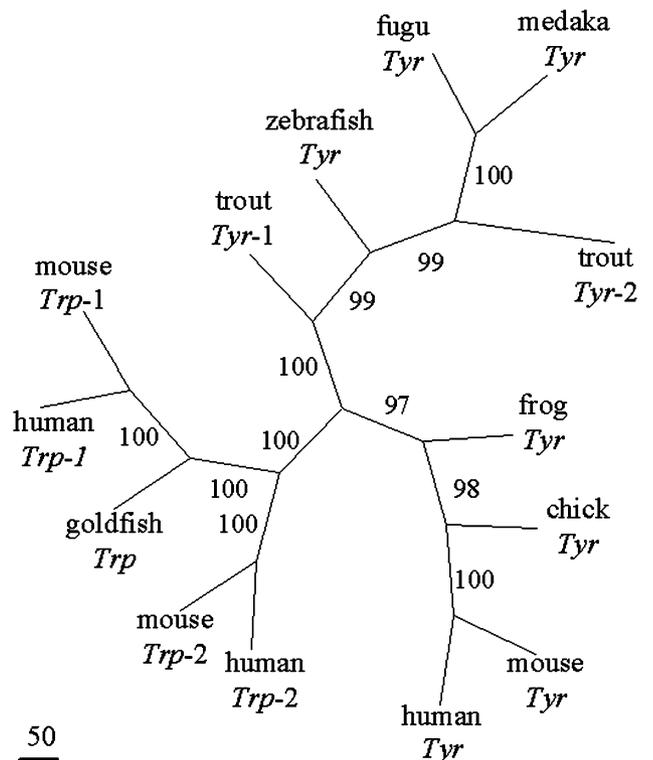


Fig. 4. Phylogenetic tree of tyrosinase and related proteins. The tree was constructed on the basis of the neighbor-joining method (15) using the deduced aa sequences listed below. The tyrosinases and related proteins that were included in the analysis are given with GenBank accession numbers. Tyrosinases (*Tyrs*): human (M27160); mouse (D00440); chicken (D88349); frog (D12514); medaka (D29687); zebrafish (AF542067); and fugu (AF343911). Tyrosinase-related proteins (*Trps*): human *TRP 1* (X51420); human *TRP 2* (D17547); mouse *Trp1* (X03687); mouse *Trp2* (X63349); and goldfish *Trp* (S71755). The scale bar indicates the genetic distance. Note that the rainbow trout *Tyr-1* and *Tyr-2* aa sequences belonged to the tyrosinase branch.

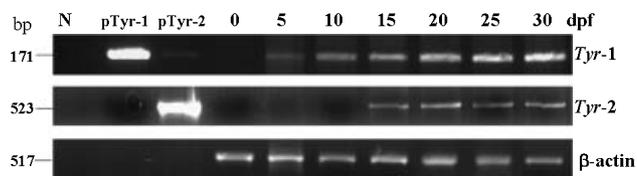


Fig. 5. RT-PCR of *Tyr-1* and *Tyr-2* transcripts in wild-type embryos at various developmental stages. cDNAs were synthesized from total RNA extracted from embryos at various dpf and were used for PCR. The negative control used distilled water. pTyr-1 was the plasmid DNA containing the full length *Tyr-1* cDNA. pTyr-2 was the plasmid DNA containing the full length *Tyr-2* cDNA. Note that *Tyr-1* expression is first detected in embryos at 5 dpf, whereas *Tyr-2* expression is first detected at 15 dpf.

Expression of Tyrosinase Genes in Early Embryos and Albino Mutants

To measure tyrosinase gene expression during embryogenesis, we performed RT-PCR of *Tyr-1* and *Tyr-2* in wild-type embryos at various developmental stages. *Tyr-1* expression was first detected at 5 dpf, whereas *Tyr-2* expression was not detected until 15 dpf (Fig. 5).

The tyrosinase activities of both dominant and recessive albino mutants were much lower than that of the wild-type embryos (Fig. 6A). However, semi-quantitative RT-PCR revealed that *Tyr-1* and *Tyr-2* mRNA levels in both albino mutants were similar to those of the wild-type embryos (Fig. 6B). In order to determine whether tyrosinase mRNAs that were derived from albino mutants contained large deletions, truncations or insertions, we performed RT-PCR using primers against the beginning and the end of the coding regions. Amplicons from each albino mutant had the same molecular weight as those from the wild type (Fig. 6C). Further, recessive albino *Tyr-1* and *Tyr-2* cDNAs were cloned and sequenced. Three nucleotide substitutions were occurred at nt 5, 637, and 1087 past the ATG start codon of the wild type *Tyr-1* cDNA sequence. Although nucleotide substitution at nt 637 was silent, those at nt 5 and 1087 caused amino acid changes (aa2; P to R and aa 363; L to M) (Fig. 2, pentagon boxes). In the coding region of recessive albino *Tyr-2* cDNA, six nucleotide substitutions (nt 18, 784, 893, 973, 1051, and 1392) and one nucleotide deletion (between nt 785 and 787 in the codon for L) were occurred. Although nucleotide substitutions at nt 18 was silent, that at nt 784 introduced amino acid change (aa 262; P to A) (Fig. 2, pentagon boxes). In addition, the nucleotide deletion resulted in the change of the codon for L to the termination signal, TGA, after the amino acid residue 262 (Fig. 2; open arrow).

Knockdown of Tyrosinase Gene Expression by AMOs

In wild-type rainbow trout embryo, eye pigmentation was clearly observed at around 25 dpf (Fig. 7A). Therefore, we injected AtTyr-1, AtTyr-2 or Sc-MO into wild-type embryos at one-cell stage and observed the accumulation of melanin in the retina of 25-dpf embryos. The accumulation of melanin was notably reduced in embryos that received either AtTyr-1 or AtTyr-2 (Fig. 7B and C), whereas Sc-MO had no effect (Fig. 7D). Furthermore, to investigate any synergistic effects, we co-injected both AtTyr-1 and AtTyr-2 (each at

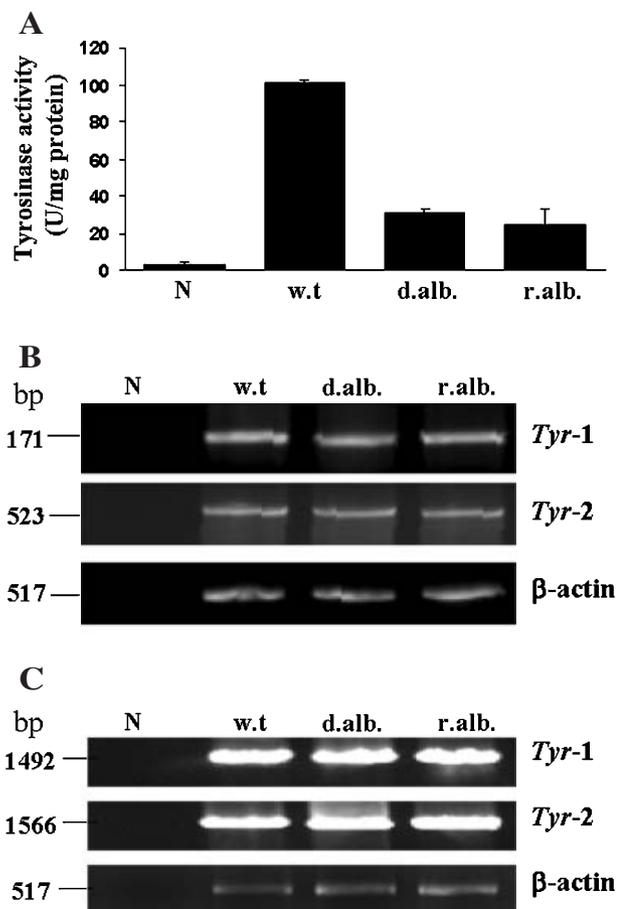


Fig. 6. Tyrosinase gene expression in wild-type, dominant albino and recessive albino embryos. (A) Tyrosinase enzymatic activity in wild type and albino mutants. The enzymatic activities were calculated from the DOPA reaction, measured using the colorimetric method. Data represents the means \pm standard error (SE) from at least three independent experiments performed in triplicate. (B) Semi-quantitative RT-PCR analysis of *Tyr-1* and *Tyr-2* transcripts in wild-type (w.t.), dominant albino (d.alb.) and recessive albino (r.alb.) mutants using mRNA extracted from 10 pooled head samples of newly hatched rainbow trout embryos. RT-PCR with β -actin primers was performed as an internal control. (C) RT-PCR analysis of the entire coding region of the tyrosinase gene transcripts. The negative controls (N) used distilled water instead of crude protein (A) or template cDNAs (B and C).

2.5 ng/embryo) into embryos. This resulted in decreased melanin accumulation, although levels were similar to those of embryos that received either AtTyr-1 or AtTyr-2 at 5 ng/embryo (data not shown). Tyrosinase activity was measured to confirm the effects of the AMOs. The enzyme activities of embryos injected with AtTyr-1 or AtTyr-2 were significantly lower than those of uninjected embryos (Fig. 8). Similarly, co-injection of both AtTyr-1 and AtTyr-2 (each at 2.5 ng/embryo) significantly reduced tyrosinase activity. By contrast, the tyrosinase activity of Sc-MO-injected embryos did not differ from that of uninjected embryos.

We further examined the effects of AMOs on melanogenesis using newly hatched wild-type embryos. Melanophores could be clearly observed in the skin of newly hatched embryos (that is, after 30 dpf) (Fig. 7F). We therefore examined the effects of AMOs on the accumulation of

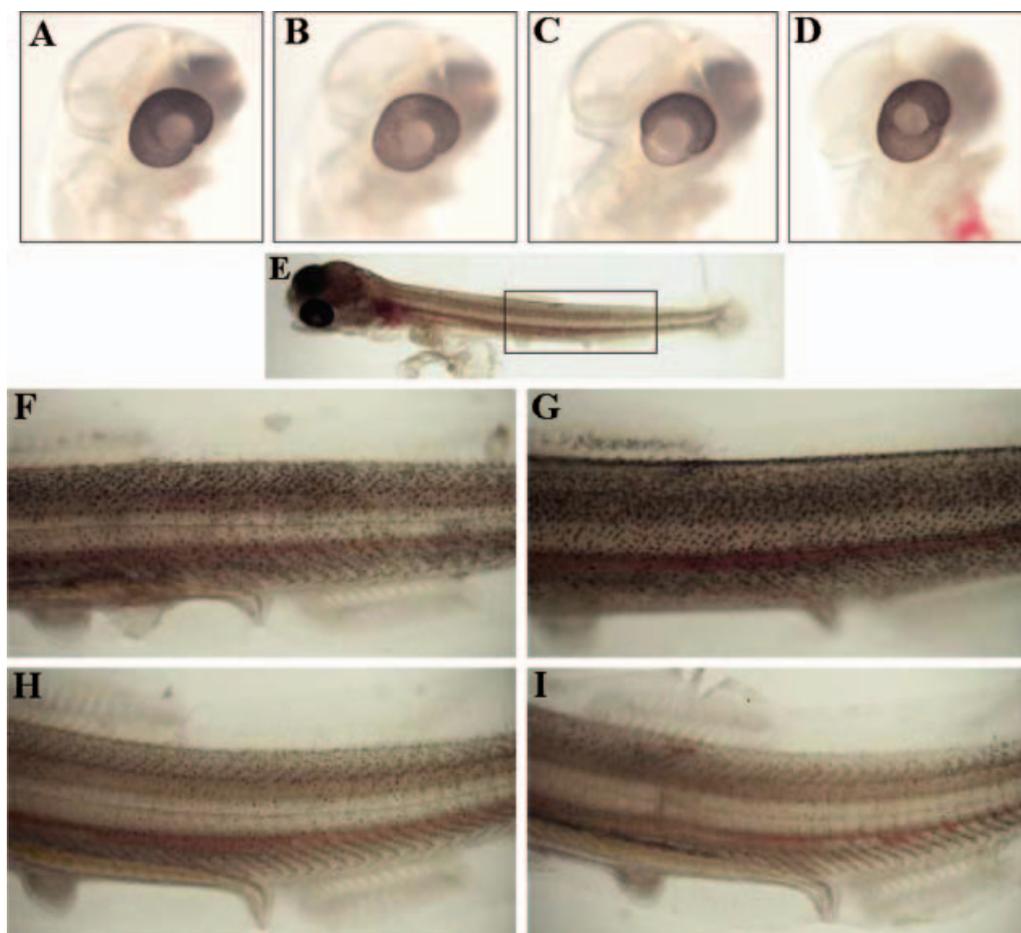


Fig. 7. Inhibition of tyrosinase gene expression by MOs. The figure shows tyrosinase gene expression in 25-dpf (A–D) and 35-dpf embryos (F–I) that were treated with MOs (5 ng/embryo). (A) The accumulation of melanin in the retina of an uninjected wild-type embryo. Injection of AtTyr-1 (B) or AtTyr-2 (C) at 5 ng/embryo led to reduced melanin accumulation. By contrast, the injection of Sc-MO did not have an effect on eye pigmentation (D). (E) A newly hatched embryo; the boxed area is magnified in panels F–I to show the pattern of skin pigmentation of an uninjected wild-type embryo (F), a Sc-MO-injected embryo (G), an AtTyr-1-injected embryo (H) and an AtTyr-2-injected embryo (I). Note that melanin accumulation was noticeably reduced in the retina and the skin of both AtTyr-1- and AtTyr-2-injected embryos.

melanin in the retina and skin of 35-dpf embryos. At this stage, it was not possible to observe differences in the accumulation of melanin in the retina of uninjected wild type and MO-treated embryos (data not shown). However, the injection of AtTyr-1 or AtTyr-2 (each at 5 ng/embryo) notably reduced melanin accumulation in the skin (Fig. 7G and H). By contrast, no effects were observed in embryos that were injected with Sc-MO at the same dose. AtTyr-1- or AtTyr-2-injected embryos therefore had significantly lower enzymatic activities in both the head and body compared with those of uninjected wild-type embryos (Fig. 8). Co-injection of both AtTyr-1 and AtTyr-2 (each at 2.5 ng/embryo) significantly reduced the activity of tyrosinase. By contrast, no effects were seen in embryos that were injected with Sc-MO.

DISCUSSION

Two types of tyrosinase cDNA, *Tyr-1* and *Tyr-2*, were cloned from the rainbow trout. Both contained motifs that were predicted to code for previously reported structural features

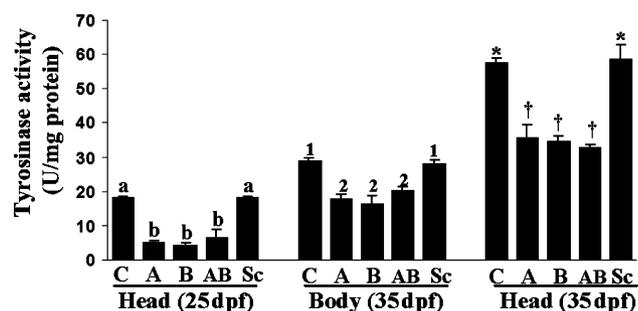


Fig. 8. Inhibitory effects on tyrosinase enzymatic activity by AMOs. DOPA assays were performed to examine the effects of *Tyr-1* and/or *Tyr-2* gene knockdown. For 25-dpf embryos, 50 head samples were pooled for protein extraction. For 35-dpf embryos, 25 head samples and 15 body samples were pooled for protein extraction. The untreated control is designated as C. Embryos in groups A, B and Sc were injected with AtTyr-1, AtTyr-2 and Sc-MO, respectively, at a dose of 5 ng/embryo. Embryos in group AB were co-injected with AtTyr-1 and AtTyr-2, each at a dose of 2.5 ng/embryo. The data are the mean \pm standard error (SE) from at least three independent experiments performed in duplicate. The letters denote statistically significant differences at $P < 0.01$.

of tyrosinase. Two hydrophobic domains were predicted that corresponded to the signal peptide and transmembrane domain (21). Most of the cysteine residues that are crucial for the formation of the tyrosinase secondary structure were predicted at the relevant positions. Consensus sequences of copper-binding sites, which are important for catalytic activity (23), were also predicted, and four or five potential *N*-glycosylation sites were present in *Tyr-1* and *Tyr-2*, respectively. In addition, *Tyr-1* and *Tyr-2* were predicted to contain four distinct domains (I–IV), which are conserved among all known tyrosinases: domains I and III, which span the first and second cysteine-rich regions, and domains II and IV, which span the histidine-rich regions (21).

By PCR analysis using genomic DNA of a homozygous gynogenetic embryo, we could obtain amplicons encoding *Tyr-1* and *Tyr-2*, demonstrating that *Tyr-1* and *Tyr-2* were located in different loci but not in different alleles of rainbow trout genome. Phylogenetic analysis also indicated that *Tyr-1* and *Tyr-2* belonged to the tyrosinase family. Therefore, the predicted topology, consensus sequences and sequence homology of rainbow trout *Tyr-1* and *Tyr-2* matched the expected structural traits of a tyrosinase. In addition, the association of both rainbow trout *Tyr-1* and *Tyr-2* with medaka and zebrafish tyrosinase genes was the closest among the vertebrate genes studied, which indicates that protein structure is highly conserved among the teleosts.

The assay revealed a reduction in tyrosinase activity associated with albinism in both dominant and recessive albino mutants. Semi-quantitative RT-PCR analysis, however, revealed that the levels of *Tyr-1* and *Tyr-2* transcripts in both types of albino mutant were similar to those of wild-type embryos. Furthermore, the transcripts of both mutants contained coding regions of the same length as those of wild-type embryos. Sequenced analysis of *Tyr-1* cDNA derived from recessive albino mutant revealed that two amino acid substitutions were found compared with that of the wild type. These two amino acid substitutions were found in the region of predicted signal peptide and domain IV (21). The domain IV is one of the well-conserved regions for all known tyrosinases and expected to be functionally important. Although we could not conclude whether these mutations caused serious effects on enzyme activity in this study, it is quite possible that these amino acid substitutions in *Tyr-1* reduce enzymatic activity. In addition, one amino acid substitution was found in *Tyr-2*. Moreover, one nucleotide deletion of recessive albino *Tyr-2* cDNA introduced the stop codon after the amino acid residue 262. Wild-type *Tyr-2* cDNA, consisting of 513 amino acids, has two copper-binding regions (Fig. 2), however, the truncated tyrosinase consisting of 262 amino acids and lacking one potential copper binding regions may be catalytically inactive. Thus, it is possible that the reduced enzyme activities in recessive albino might have been caused by point mutations of *Tyr-1* and truncation of *Tyr-2*. In humans, many reports have shown that the deletion, insertion or substitution of just a few nucleotides in the tyrosinase gene can cause frameshift or aa substitutions. Such mutants express the tyrosinase gene without detectable changes in either the amount or size of the gene transcripts, although they show reduced or absent tyrosinase activity in vivo (24).

Dominant albinism is rare in the rainbow trout and we were unable to identify its cause in this study. If albinism is caused by the mutation of pigmentation genes, such as tyrosinase genes, the phenotype is generally recessively inherited. Using an intragenic microsatellite marker for *Tyr-1*, we have previously shown that the causative locus for dominant albinism does not correspond with the locus of *Tyr-1* (25). It is possible that dominant albinism is the result of other factors that inhibit tyrosinase activity. In humans, dominant albinism has been reported that is caused by a dominant-negative mutant allele of the c-KIT membrane-receptor gene (26, 27). In addition, zebrafish c-kit has been shown to be required for morphogenesis of a subpopulation of melanocytes, which indicates that it has a crucial role in melanin biosynthesis in fish (28). Recently, a number of genes, including *Wnt*, *Sox10*, *Mitf*, *Ednrg/Et3*, and *Fms/Csf1*, have been revealed to induce pigment pattern mutations in zebrafish (29). Therefore, analyses of orthologs of above mentioned genes using dominant albino trout would also be important.

Gene knockdown using AMOs has been shown to be a powerful tool for the investigation of gene function in *Xenopus* (30), zebrafish (31) and rainbow trout (32). In this study, we used this technique to examine the function of *Tyr-1* and *Tyr-2* gene products in wild-type embryo. The onset of *Tyr-1* expression began at 5 dpf and increased around 10–15 dpf, and *Tyr-2* expression was initiated at 15 dpf. This coincides with the initiation of eye pigmentation in rainbow trout embryos. Treatment with AMOs resulted in reduced melanin accumulation in embryos at both 25 and 35 dpf. These results indicate that *Tyr-1* and *Tyr-2* both have crucial roles in melanin synthesis. Moreover, the significant decline of tyrosinase enzymatic activity in these embryos confirmed that the reduced melanin accumulation was caused by the decline of tyrosinase activity. There was no significant difference between embryos injected with AtTyr-1 and those injected with AtTyr-2, which suggests that *Tyr-1* and *Tyr-2* have similar roles in melanin biosynthesis. The co-injection of both AtTyr-1 and AtTyr-2 also reduced tyrosinase activity, but the effects were similar to those of either AMO on its own. This might be the result of the lower doses of each AMO (2.5 ng/embryo) in the co-injected embryos – the maximum amount of AMO that could be administered without malformation was a total of 5 ng/embryo, so we could not increase the dose of each AMO. Indeed, it is known that the rainbow trout has a tetraploid origin (33) and the existence of two isoforms of the tyrosinase gene without functional differences might be partly explained by this fact. In general, it has been believed that an isoform emerged by gene duplication degenerates gradually during evolution. However, recent studies suggested that some duplicated genes would be preserved by obtaining novel functions or changing their expression patterns (34). Therefore, the existence of two functional tyrosinase genes in rainbow trout might be at an early stage of their functional divergence leading to a more complex pigmentation system.

Gene knockdown by AMOs is based on the simple principle of inhibiting the expression of a target gene by preventing the binding of ribosomes to the target mRNA. As a result, the translation process is inhibited; that is, the

AMOs interfere with tyrosinase gene expression at the level of translation. Using optimal doses, AMOs have been widely reported to cause the sequence-specific inhibition of gene expression without toxicity in living cells (35). In this study, the use of AMOs against tyrosinase genes interfered with melanin biosynthesis. AMOs could therefore be a powerful depigmenting agent for the treatment of acquired skin hyperpigmentation. Several novel methods have recently been developed to introduce AMOs into living cells (35). Therefore, a combination of these new delivery methods and AMOs against tyrosinase genes might lead to the development of innovative whitening techniques. Most depigmenting agents reduce the function of tyrosinase enzymes through interference with transcription, post-transcriptional control or glycosylation (36). This is the first report to demonstrate the inhibition of melanin synthesis by blocking tyrosinase gene expression at the level of translation.

In this study, we cloned two types of tyrosinase gene from rainbow trout. These genes might be valuable targets for the development of novel gene knockdown and knockout techniques in rainbow trout. In addition, they represent potential candidate reporter genes in combination with recessive albino mutants. Further studies will be necessary to reveal the details of the mechanisms of rainbow trout albinism.

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