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Generation of Albino *Cynops pyrrhogaster* by Genomic Editing of the *tyrosinase* Gene

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Albino animals are useful for in situ hybridization experiments that demonstrate gene expression in embryos and organs, for the immunological rejection of skin grafts transplanted to host animals, and to identify tissues with regenerative ability during limbs and retina regeneration processes. *Cynops pyrrhogaster* has extensive regenerating capacities. To facilitate regenerative research, in the present study, we produced albino *C. pyrrhogaster* using genomic editing. The DNA fragment containing part of the *tyrosinase* gene from *C. pyrrhogaster* was amplified using degenerate primers corresponding to evolutionarily conserved nucleotide sequences among several species, and the nucleotide sequence was determined. We designed a transcription activator-like effector nuclease (TALEN) that targets a candidate of the *C. pyrrhogaster tyrosinase* gene. Fertilized eggs were injected with TALEN mRNA, and albinos of *C. pyrrhogaster* were obtained. The results of the present study demonstrated that TALEN can be used effectively for genomic editing in *C. pyrrhogaster* and that the candidates of the *tyrosinase* gene that were cloned by us are essential for melanin synthesis. The albino newts created in the present study can be used as versatile experimental material.

Key words: *Cynops pyrrhogaster*, *tyrosinase*, TALENs, genomic editing, targeted gene knockout, albino phenotype

INTRODUCTION

The albino phenotype is important and useful for many experiments including in situ hybridization and transplantation of nuclei, skin, and organs. John Bertrand Gurdon transplanted the nuclei from a single tail-bud albino embryo of *Xenopus laevis* into u.v.-enucleated unfertilized eggs of the wild-type female and produced 30 frogs, which were all female and albino. These frogs were the first vertebrate clones generated using nuclear transplantation techniques. This study also indicated that the nuclei of a tail-bud embryo contain the genetic information that is necessary to form all types of differentiated somatic cells in the adult frogs (Gurdon, 1962, 1977). Recently, the following albino amphibians have been developed: *Xenopus tropicalis* (Ishibashi et al., 2012; Nakajima et al., 2012), *Xenopus laevis* (Nakajima and Yaoita, 2015b; Suzuki et al., 2013) and *Pleurodeles waltl* (Hayashi et al., 2014). These albinos were produced by modifying the *tyrosinase* gene using zinc-finger nucleases or transcription activator-like effector nucleases (TALENs). Tyrosinase is essential for melanin biosynthesis, and it converts tyrosine to dopaquinone in the initial step of the melanin synthesis pathway.

Urodeles are recognized for the regenerative ability of their limbs, tail, brain, and heart. In particular, *Cynops pyrrhogaster*, one of the most abundant species of newts in Japan, effectively regenerates limbs (Asahina et al., 1999; Kato et al., 2003; Shimizu-Nishikawa et al., 2001), jaw (Kurosaka et al., 2008), lenses (Inoue et al., 2012; Okamoto et al., 1998; Okamoto et al., 2004), and retinas (Fujisawa, 1981; Kaneko and Saito, 1992; Nakamura et al., 2014). Moreover, experimental techniques such as transgenesis have been established in *C. pyrrhogaster* (Casco-Robles et al., 2011).

In the present study, we showed that albino *C. pyrrhogaster* can be generated by modifying a tentative *tyrosinase* gene that was obtained by gene amplification using a pair of primers corresponding to the evolutionary conserved nucleotide sequences of *tyrosinase* genes. These albino newts represent good material for studies of regeneration, transplantation, and pigmentation.

MATERIALS AND METHODS

Animals

Cynops pyrrhogaster newts were caught in the field and raised in the laboratory for more than two years at 20–24°C. The ovulation and breeding of embryos were performed as previously described (Casco-Robles et al., 2011). All of the newts were maintained and used following the guidelines established by Hiroshima University for the care and use of experimental animals.

Cloning of the *C. pyrrhogaster tyrosinase* gene

A tail tip was placed in 800 µl 50 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$. After the addition of 50 µl 10% SDS and 30 µl 10 mg/ml proteinase K, the mixture was incubated at 65°C for several hours. The genomic DNA was extracted using phenol and chloroform. An amount of 1 µg DNA was used as a substrate in polymerase chain reaction (PCR) amplification by a three-step protocol [(95°C, 30 s; 65°C, 30 s; 72°C, 30 s) × 40]; the degenerate primers

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(10 μM of 5′-GGMGAYGRKTCYYCTTGTGGNSAG-3′ and 5′-AAARSMHGGDGCTTCRTGRGCAAA-3′) (Supplementary Figure S1 online) and TaKaRa *Ex Taq* Hot Start Version (TaKaRa) were used in the reaction. The nucleotide sequence of the amplified DNA fragment was determined to clone the full-length cDNA sequence using the 5′ and 3′ rapid amplification of cDNA ends (Frohman et al., 1988). The full-length cDNAs were cloned by PCR using a pair of primers, full-lengthF and full-lengthR, that were located upstream and downstream of the coding region, respectively (Supplementary Figure S1 online).

TALEN construction

DNA-binding domains were designed to target the sequences 5′-CGCCGATATCAGCGTCTACGA-3′ and 5′-CCCGGGACGCG-

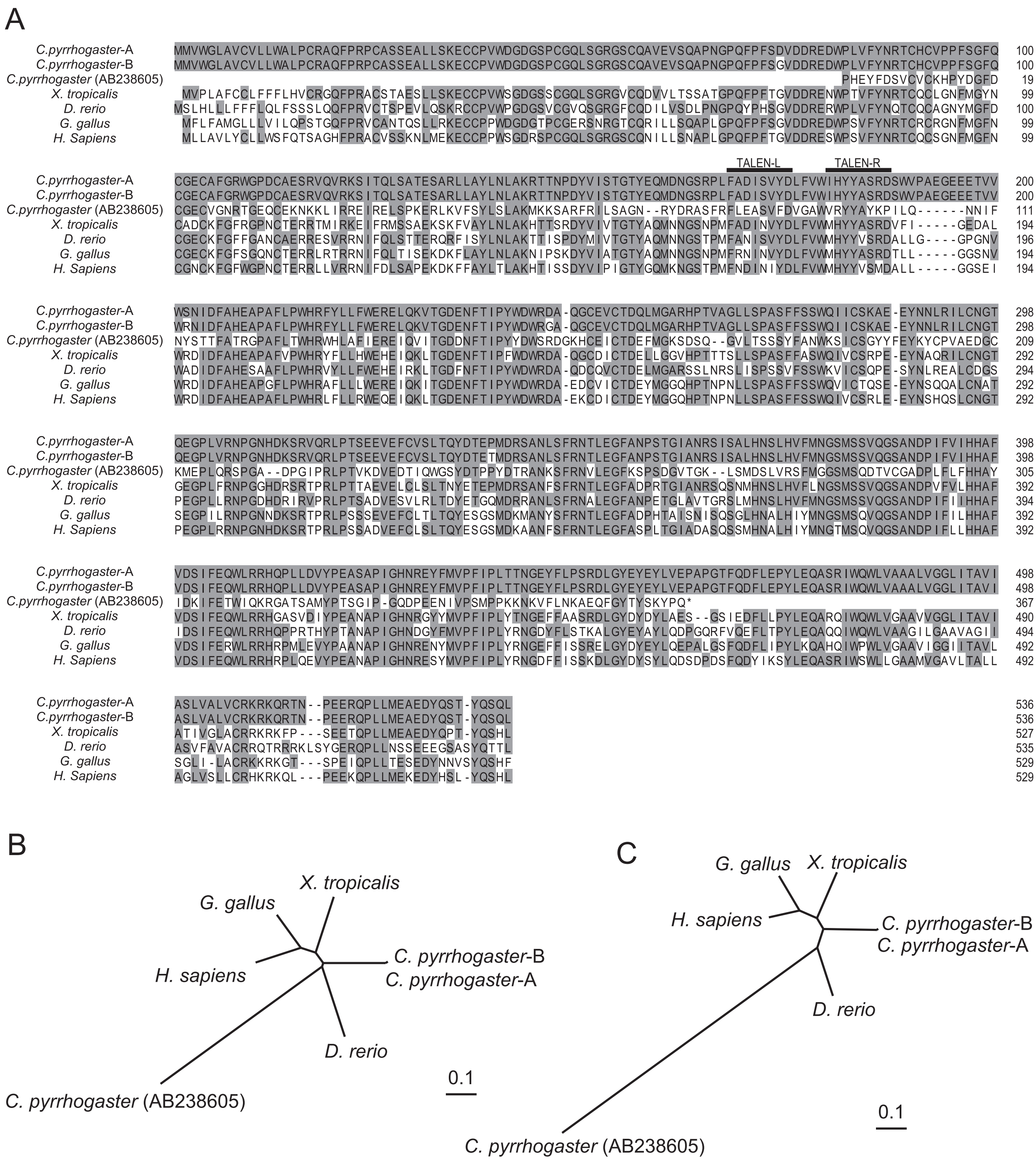

Fig. 1. Comparison of tyrosinase sequences. **(A)** Alignment of amino acid sequences that were deduced from the nucleotide sequences of *Cynops pyrrhogaster tyrosinase*-A [DDBJ:LC076439] and -B [DDBJ:LC076440], *C. pyrrhogaster tyrosinase* [DDBJ:AB238605], *Xenopus tropicalis tyrosinase* [GenBank:BC135591], *Danio rerio tyrosinase* [JPO:E0128319], *Gallus gallus tyrosinase* [DDBJ:D88349.1], and *Homo sapiens tyrosinase* [GenBank:M27160.1]. Shaded boxes indicate amino acids that are the same as those in *C. pyrrhogaster tyrosinase*-A. Cp-Tyr-TALEN target sites are denoted by solid bars. **(B, C)** Phylogenetic trees of tyrosinase cDNA **(B)** and amino acid sequences **(C)**. Phylogenetic relationships were deduced using the neighbor-joining method. The scale bar indicates the number of nucleotide or amino acid substitutions per site.

TAGTAGTGA-3′ (Fig. 2). TALEN repeats were assembled as previously described (Cermak et al., 2011), with minor modifications (Nakajima et al., 2013) and were inserted into pTALEN-ELD-DS and pTALEN-KKR-DS (Nakajima and Yaoita, 2015a) to generate the Cp-Tyr-TALEN expression constructs.

RNA microinjection

mRNA was transcribed from the *Xba*I-digested Cp-Tyr-TALEN expression constructs in vitro using the mMESSAGE mMACHINE SP6 kit (Ambion).

Each Cp-Tyr-TALEN mRNA (4 nl; 50 ng/μl) was dissolved in nuclease-free water (Ambion) and injected into *C. pyrrhogaster* fertilized eggs suspended in 6% Ficoll PM 400 (Sigma)/0.5 × MHS (Casco-Robles et al., 2011).

DNA extraction

A single embryo that was injected with Cp-Tyr-TALEN-mRNAs was homogenized in 270 μl 50 mM NaOH and incubated for 10 min at 95°C. The homogenate was neutralized with 30 μl 1 M Tris-Cl (pH 8.0) and centrifuged at 20,400 × *g* for 5 min at 4°C. The supernatant was then extracted using phenol and chloroform.

Mutation analysis

A DNA fragment containing the target sites was amplified using KOD FX Neo (TOYOBO) and the primers 5′-GTTTCCGTTTTCG-GATGTGGACGA-3′ and 5′-GTCGATGTTACTCCACACCACAGT-3′. The reaction conditions were as follows: pre-denaturation (94°C, 120 s) followed by a three-step protocol [(98°C, 10 s; 65°C, 30 s; 72°C, 30 s) × 40]. The second round of PCR was performed using EmeraldAmp MAX PCR Master Mix (TaKaRa) and the same primers with a three-step protocol [(95°C, 30 s; 65°C, 30 s; 72°C, 30 s) × 10]. The amplicon was ligated into the pGEM-T Easy vector (Promega), and the nucleotide sequences were subsequently determined.

qPCR

Total RNA was purified from a tail tip using the SV Total RNA Isolation System kit (Promega). Sample RNA was reverse transcribed using the ReverTra Ace qPCR RT Master Mix (TOYOBO). Diluted product (2 μl) was subjected to qPCR using a SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (TaKaRa) in 20 μl of reaction solution. qPCR was performed using a Thermal Cycler Dice Real-Time System (TaKaRa). The reaction conditions included pre-denaturation (95°C, 30 s) and a two-step protocol [(95°C, 5 s; 60°C, 30 s) × 40]. The results were analyzed using a Thermal Cycler Dice Real-Time System Ver. 4.00 (TaKaRa). The level of specific mRNA was quantified and normalized to the amount of sample RNA. The primer sequences used for the amplification were 5′-AGTGCCAGATTCCGGATACT-CTCT-3′ and 5′-CTGGTGGCAAACGT-GGTGGAATAA-3′.

RESULTS AND DISCUSSION

The sequence of the *C. pyrrhogaster tyrosinase* gene was previously deposited in the GenBank database [DDBJ:AB238605]. However, the similarity of this gene to *tyrosinase* gene sequences obtained from other species is low at the nucleotide (Fig. 1B, Supplementary Figure S1 online) and amino acid (Fig. 1A, C) levels. To verify whether the deposited sequence is a *tyrosinase* gene of *C. pyrrhogaster*, we amplified a DNA fragment by PCR using degenerate primers that were designed based on the regions that are conserved between *X. laevis* [GenBank: AY333967.1], *X. tropicalis* [GenBank:BC135591], *Hymenochirus boettgeri* [GenBank:AY341763.1], *Opisthocomus hoazin* [GenBank:XM_009932562.1], and *Pelecanus crispus* [GenBank:XM_009480710.1] *tyrosinase* gene sequences (Supplementary Figure S1 online). The amplicon was subcloned, and the sequences of the clones were determined. Full-length cDNA was cloned by standard molecular cloning methods using the amplicon sequence. Sequence analysis revealed that two *tyrosinase* genes, Cp *tyrosinase*-A [DDBJ:LC076439] and -B [DDBJ:LC076440], have 99.2% and 99.3% identities at the nucleotide (Supplementary Figure S1 online) and amino acid (Fig. 1A) levels, respectively. The gene trees of *tyrosinase* cDNA and amino acid sequences clearly indicate that the sequences of Cp *tyrosinase*-A and -B are more similar to the *tyrosinase* sequences of other species than the reported sequence [DDBJ:AB238605] (Figs. 1B, C).

Fig. 2. Site-directed mutagenesis in *Cynops pyrrhogaster* embryos injected with Cp-Tyr-TALEN mRNAs. The target DNA fragment was amplified using genomic DNA samples that were purified from one 7-day-old and one 32-day-old larvae (#1 and #2) and re-cloned for sequence determination. Wild-type nucleotide and amino acid sequences are indicated at the top and bottom of the panel, respectively. A pair of solid bars denotes the Cp-Tyr-TALEN-binding sites. Gaps resulting from a deletion (Δ) are indicated by dashes. Nucleotides that differ from the wild-type (WT) sequence are indicated in bold and underlined characters. The mutation types are indicated on the right. The ratio of the number of the indicated sequence to the total number of sequences is shown in parentheses on the right.

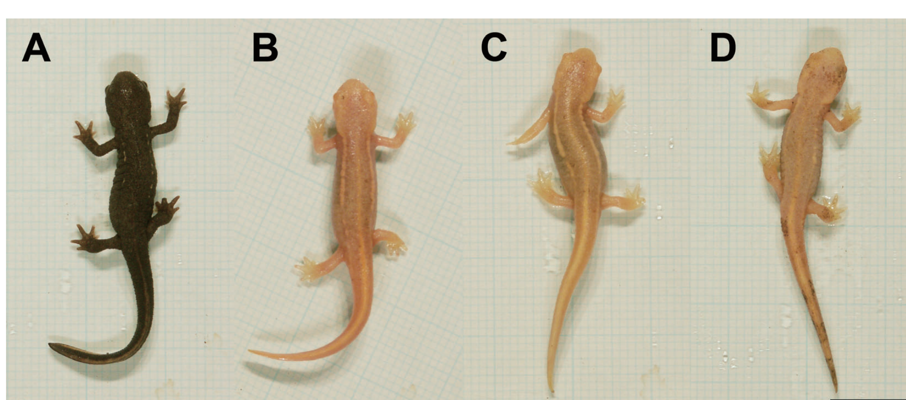

Fig. 3. Albino newts generated from knockout of the *tyrosinase* gene. **(A)** A wild-type 4.5-month-old newt. **(B–D)** Albino 4.5-month-old newts that were generated by injecting Cp-Tyr-TALEN mRNAs at the one-cell **(B, C)** and two-cell **(D)** stages. Scale bar is 10 mm.

To examine whether Cp *tyrosinase*-A and -B are essential for melanin synthesis, we designed TALENs (designated Cp-Tyr-TALENs) that targeted the common region of Cp *tyrosinase*-A and -B (Fig. 2, Supplementary Figure S1 online). Fertilized eggs were injected with mRNA that was synthesized using Cp-Tyr-TALENs. Genomic DNA was purified from one 7-d-old larva and subjected to mutation analysis. All eight clones harbored mutations. Seven of eight clones contained an out-of-frame mutation, and one clone had an in-frame mutation (Fig. 2). Mutation analysis using one 32-d-old larva showed that three of twelve clones contained a mutation in the target sites (25%); namely, a large deletion of 310 base pairs (bp) and an insertion of 67 bp in one clone, a 9-bp deletion and 4-bp insertion in another clone, and a 6-bp deletion in the third. Three newts developed from embryos that had been injected with Cp-Tyr-TALEN-mRNAs at the one cell stage, and two were apparent albinos (Figs. 3B and C). Only one newt survived from embryos had been injected at the two-cell stage into both blastomeres, and displayed a similar phenotype (Fig. 3D). The mutation types were determined in these apparent albino newts using genomic DNA prepared from tail tips. Among the clones, 92% (#3) and 100% (#4) had a mutation in the target sites of Cp-Tyr-TALEN; however, only 4/13 (#3) and 6/11 (#4) of the mutations were out-of-frame (Fig. 4). Their albino phenotype suggests that the *tyrosinase* function was impaired by the in-frame mutations, because almost all genes should be non-functional in pigment cells. The spacer sequence between TALEN target sites encodes evolutionarily conserved amino acid sequences (LFVW) among *C. pyrrhogaster*, *X. tropicalis*, *Danio rerio*, *Gallus gallus*, and *Homo sapiens* (Fig. 1), suggesting the important function of this region. It is also possible that almost all skin melanophores have out-of-frame mutations, whereas other cells have in-frame and out-of-frame mutations. The mutation rate and type were variable among skin samples and not correlated to the level of skin pigmentation, even if they

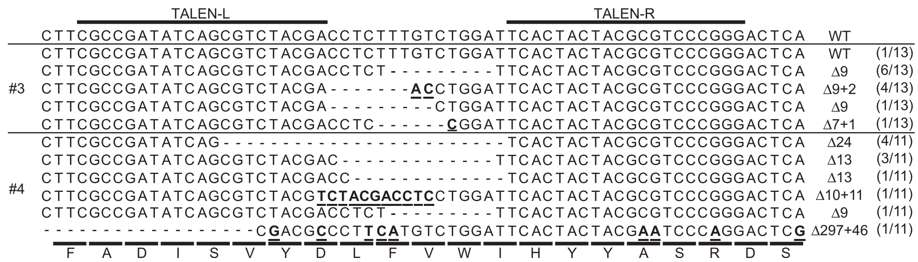

Fig. 4. Mutational analysis of the albino newts. The target DNA fragment was amplified using genomic DNA samples that were purified from the tail tip of two albino newts (Figs. 3B, C)(#3 and #4) and recloned for sequence determination. The alignment is labeled as described in the Fig. 2 legend.

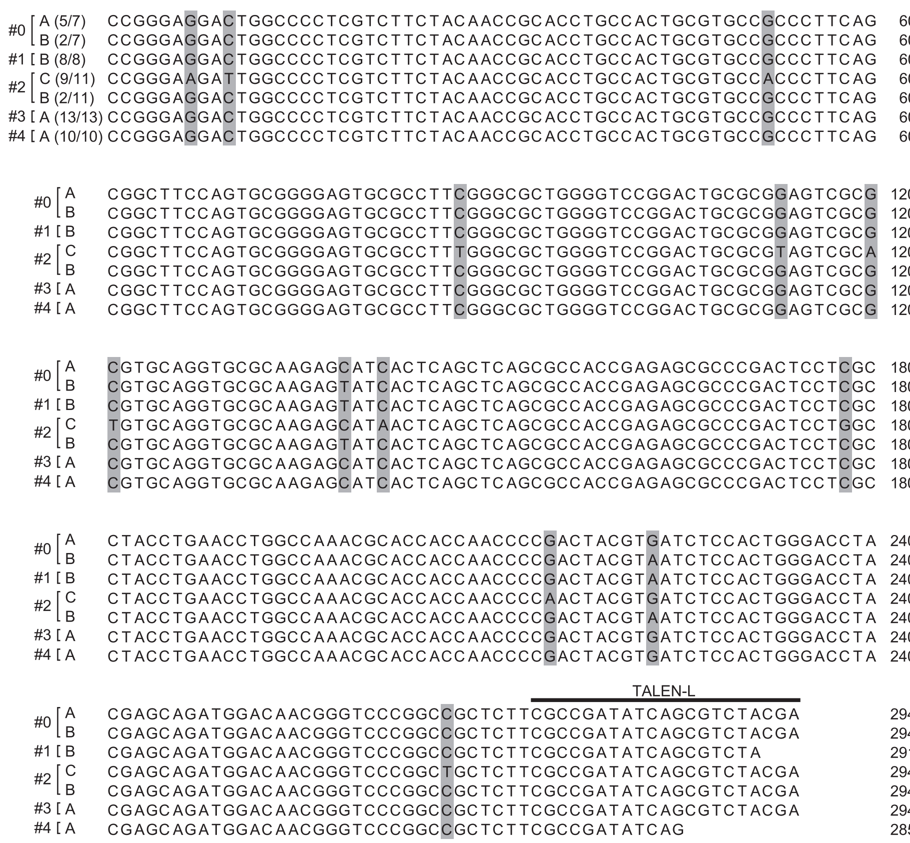

Fig. 6. Alignment of the *tyrosinase* sequences obtained from each individual. The target DNA fragment was amplified using genomic DNA that was purified from a wild-type adult newt (#0), Cp-Tyr-TALEN-mRNA-injected 7-day-old (#1) and 32-day-old (#2) embryos, and Cp-Tyr-TALEN-mRNA-injected albino newts (#3, #4). Sequences upstream of the Cp-Tyr-TALEN-binding site are compared, and divergent nucleotides are shaded. The three allotypes are denoted as A (*Cynops pyrrhogaster tyrosinase*-A), B (*C. pyrrhogaster tyrosinase*-B) and C. The ratio of the number of the indicated sequence to the total number of sequences in each individual is shown in parentheses. Mutations with a large deletion (Δ310 + 67 in #2 and Δ297 + 46 in #4) are not counted.

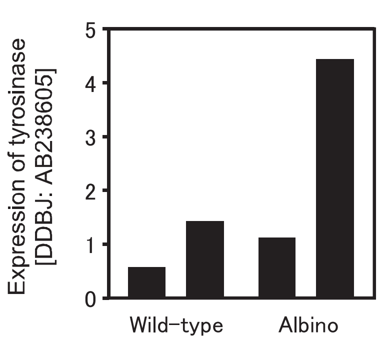

Fig. 5. Expression of tyrosinase [DDBJ: AB238605]. Expression levels of tyrosinase [DDBJ:AB238605] in the tail tips of two wild-type and two albino newts were determined by qPCR and are shown in arbitrary units.

were excised from a single chimeric F0 that had been injected with anti-tyrosinase TALEN mRNAs (Nakajima et al., 2012).

We examined the expression level and sequence (802 bp) of *C. pyrrhogaster tyrosinase* [DDBJ:AB238605] mRNA to know whether they are expressed without mutations even after the injection of the Cp-Tyr-TALEN-mRNAs. The expression levels in two albino newts were comparable to those of two wild-type newts (Fig. 5). The nucleotide sequence comparison showed more than 99% identity (99.1~99.6%) with the submitted sequence of *C. pyrrhogaster tyrosinase* [DDBJ:AB238605] in six clones obtained from wild-type newts and sixteen clones from albino newts (data not shown). Furthermore, *C. pyrrhogaster tyrosinase* [DDBJ:AB238605] was searched for Cp-Tyr-TALEN target sites using the left and right recognition sequences 5′-CRCCRATATCARCRTCTACRA-3′ and 5′-CCCRRRACRCRTARTARTRA-3′ (where R is A or G), respectively, because a TALEN DNA binding repeat that recognizes the nucleotide G also binds to the nucleotide A. There were no sequences with 14 or fewer mismatched nucleotides and 10 to 30 spacer nucleotides.

To determine whether Cp *tyrosinase*-A and -B are paralogs or allotypes, sequences upstream of the Cp-Tyr-TALEN-binding site were compared (Fig. 6). Three types of sequence were observed, and each individual had one or two sequence types; this finding strongly implies that the differences the between Cp *tyrosinase* sequences can be ascribed to allotypes.

These results demonstrated that the Cp *tyrosinase*-A and -B genes are necessary for melanin production and that the TALEN can be used effectively in *C. pyrrhogaster*. We hope that the albino form of *C. pyrrhogaster* will contribute to newt studies, including regeneration, transplantation, and pigmentation experiments.

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