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Research Article

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Efficient genome editing in medaka (*Oryzias latipes*) using a codon-optimized SaCas9 system

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Abstract: The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, belonging to the type II CRISPR/Cas system, is an effective gene-editing tool widely used in different organisms, but the size of *Streptococcus pyogenes* Cas9 (SpCas9) is quite large (4.3 kb), which is not convenient for vector delivery. In this study, we used a codon-optimized *Staphylococcus aureus* Cas9 (SaCas9) system to edit the tyrosinase (*tyr)*, oculocutaneous albinism II (*oca2*), and paired box 6.1 (*pax6.1*) genes in the fish model medaka (*Oryzias latipes*), in which the size of SaCas9 (3.3 kb) is much smaller and the necessary protospacer-adjacent motif (PAM) sequence is 5'-NNGRRT-3'. We also used a transfer RNA (tRNA)-single-guide RNA (sgRNA) system to express the functional sgRNA by transcription either in vivo or in vitro, and the combination of SaCas9 and tRNA-sgRNA was used to edit the *tyr* gene in the medaka genome. The SaCas9/sgRNA and SaCas9/tRNA-sgRNA systems were shown to edit the medaka genome effectively, while the PAM sequence is an essential part for the efficiency of editing. Besides, tRNA can improve the flexibility of the system by enabling the sgRNA to be controlled by a common promoter such as cytomegalovirus. Moreover, the all-in-one cassette cytomegalovirus (CMV)-SaCas9-tRNA-sgRNA-tRNA is functional in medaka gene editing. Taken together, the codon-optimized SaCas9 system provides an alternative and smaller tool to edit the medaka genome and potentially other fish genomes.

Key words: *Staphylococcus aureus* Cas9 (SaCas9); Medaka; Transfer RNA (tRNA); Gene editing; Tyrosinase (*tyr*); Oculocutaneous albinism II (*oca2*); Paired box 6.1 (*pax6.1*)

1 Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system is an adaptive immune system in prokaryotes (Makarova et al., 2011b; Wiedenheft et al., 2012). It was later developed as an effective tool for gene editing in multiple species due to its advantages of low cost, easy operation, and wide adaptability. The CRISPR/Cas system can be classified into three major types (types I, II, and III): type I and type III systems use multi-Cas proteins for target recognition and cleavage while the type II system needs only a single Cas protein 9 (Cas9) to achieve the effect and has become the major genome-editing tool (Makarova et al., 2011a, 2011b, 2015). Following the application of the CRISPR/Cas9 system to gene editing (Jinek et al., 2012), Emmanuelle CHARPENTIER and Jennifer A. DOUDNA were awarded the 2020 Nobel Prize in Chemistry, highlighting international recognition of its value. Notably, the Cas9 derived from *Streptococcus pyogenes* (SpCas9) has been widely applied for editing the genomes of various organisms (Dickinson et al., 2013; Gratz et al., 2013; Hwang et al., 2013). However, the genome size of SpCas9 is 4.3 kb, which is not easy to pack into the adeno-associated virus vector for therapy or editing, and the protospacer-adjacent motif

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(PAM) is 5'-NGG-3' (Mojica et al., 2009; Kleinstiver et al., 2015).

Alternative Cas9 systems have been developed, such as SaCas derived from *Staphylococcus aureus* (SaCas9), which is smaller (3.3 kb) and has a different PAM sequence (5'-NNGRRT-3'), and is therefore more suitable for packing and delivery in vivo (Friedland et al., 2015; Ran et al., 2015; Brooks and Gaj, 2018; Wu et al., 2018). Compared to SpCas9, SaCas9 using a 22-nt spacer showed higher editing efficiency and lower off-target activity in genome editing (Xie et al., 2018; Yang et al., 2022). Furthermore, the highest editing efficiency was observed using the spacer with the PAM 5'-NNGGGT-3', while the lowest was observed using the spacer with the PAM 5'-NNGGAT-3' (Yang et al., 2022). A shorter PAM sequence of SaCas9 5'-NNGG-3' was also identified on the application of SaCas9 ortholog (Hu et al., 2020). In addition, the SaCas9 system was able to directly edit the genome, including knockout or gene replacement, in different animal models (Feng et al., 2016; Zhang et al., 2016; Balboa et al., 2017; Wang et al., 2018, 2020; Katayama et al., 2019). However, successful use of the SaCas9 system in the fish model medaka has not yet been reported.

A successful gene-editing system needs a functional Cas protein and single-guide RNA (sgRNA), and expression of the sgRNA is still a limiting step in different species due to promoter conservation. The discovery of transfer RNA (tRNA) in both bacterial polycistronic transcriptional units and eukaryotes has shown that tRNA-processing systems can produce different small RNAs (Nakaar et al., 1994), while the conserved sequence makes tRNA a universal platform for accurate sgRNA processing (Xie et al., 2015). Compared with the U6 promoter, tRNA is smaller and more suitable for adeno-associated viruse (AAV) vector-based genome editing (Mefferd et al., 2015), and is a potential transcription enhancer of RNA polymerase III (Pol III) to improve the editing efficiency of the CRISPR/Cas9 system (Xie et al., 2015). Moreover, the editing ability of a tRNA-sgRNA system has been demonstrated in multiple species, including plants (Qi et al., 2016; Ma et al., 2019), mammals (Swiech et al., 2015; Dong et al., 2017), microorganisms (Numamoto et al., 2017; Zhang et al., 2019), *Drosophila* (Port and Bullock, 2016), zebrafish (Shiraki and Kawakami, 2018), and medaka (Pan et al., 2022). Therefore, the combination of the smaller SaCas9 and tRNA-sgRNA is also a desirable approach for gene editing in different species such as the medaka fish model.

The medaka is an excellent fish model, not only for functional genomics, developmental biology, and immunology, but also for testing gene function in molecular breeding for aquaculture. We have established an SpCas9 gene-editing system in medaka (Fang et al., 2018; Pan et al., 2022). Here, we examined the editing efficiency of a codon-optimized SaCas9 system to mutate three genes including tyrosinase (*tyr*), oculocutaneous albinism II (*oca2*), and paired box 6.1 (*pax6.1*) in medaka, and observed the change in phenotypes and genetic modification by sequencing.

2 Materials and methods

2.1 Medaka husbandry and breeding

Medaka fish of the Hd-rR strain were maintained at 28 °C in a water circulation system with a photoperiod of 14-h light:10-h dark (Chen et al., 2017; Pan et al., 2022). Embryos were cultured in embryonic rearing medium until the larvae hatched.

2.2 Plasmid construction

Based on the SaCas9 protein sequence (Ran et al., 2015), we optimized the DNA sequence in medaka and defined it as mSaCas9. The coding sequence of mSaCas9, a hemagglutinin (HA)-tag sequence, was inserted into pcDNA3.1 to form plasmid pC322mSaCas9, which was synthesized by GenScript Biotech Corp., Piscataway, NJ, USA (Seq. S1). The mSaCas9 messenger RNA (mRNA) was synthesized using the mMESSAGE mMACHINE T7 transcription kit (Thermo Fisher Scientific, Waltham, MA, USA).

According to the SaCas9 sgRNA scaffold sequence (Ran et al., 2015; Feng et al., 2016), the target fragment sgRNA scaffold was annealed using a pair of specific primers cRNA F/cRNA R (Table 1, Figs. 1a and 1b). The target fragment sgRNA was amplified by polymerase chain reaction (PCR) using primers in-gRNA F/Reverse (Table 1, Fig. 1b), and then inserted into the pCS2-tRNA plasmid which was digested by *Bbs*I and *Xho*I (Pan et al., 2022) (Seq. S2 and Fig. S1). The PCR programs were set as 94 °C for 3 min, followed by 35 cycles of 94 °C for 25 s, 62 °C for 30 s, and 72 °C for 15 s, and then 72 °C for 3 min. The recombinant plasmid pCS2+tRNA-mSatyr-sgRNA was sequenced (Fig. 1c, Seq. S3). Based on the tRNA sequence (Pan

Table 1 Primers used in the experiment

Primer	Sequence (5'→3')
cRNA F	GTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAGGCAAAATGCCGTG
cRNA R	AAAAATCTCGCCAACAAGTTGACGAGATAAACACGGCATTTTGCCTTG
in-gRNA F	TCGATTCCCGGCTGGTGCAGGGTCTTCGAGAAGACCTGTTTTAGTACTCTGGAAACAG
in-tRNA F	GTTGGCGAGATTTTTCTCGAGAACAAAGCACCAGTGGTCTAGTG
in-tRNA R	TATCATGTCTGGATCTACGTATGCACCAGCCGGGAATCGAAC
T7-R4445-cRNA	TAATACGACTCACTATAGGaattgtggcgatcaaagcaccGTTTTAGTACTCTGGAAAC
T7-F4553-cRNA	TAATACGACTCACTATAGGctgttgcagagcagttcagaaGTTTTAGTACTCTGGAAAC
T7-R4414-cRNA	TAATACGACTCACTATAGGgatctgctgggcttgctgaaggtaGTTTTAGTACTCTGGA
T7-R4468-cRNA	TAATACGACTCACTATAGGaacaattgtggcgatcaaagcaccGTTTTAGTACTCTGGA
T7-NR4443-cRNA	TAATACGACTCACTATAGGaggatcccggcccccaggagccaGTTTTAGTACTCTGGA
T7-NR4475-cRNA	TAATACGACTCACTATAGGccgcggcaacaattgtggcgatcaGTTTTAGTACTCTGGA
T7-R14069-cRNA	TAATACGACTCACTATAGGcgcgattttggcgaccacctcGTTTTAGTACTCTGGAAAC
T7-R14135-cRNA	TAATACGACTCACTATAGGcccctccgacaggagcctgtcGTTTTAGTACTCTGGAAAC
T7-F9011-cRNA	TAATACGACTCACTATAGGtccagaccgagacaacccctgGTTTTAGTACTCTGGAAAC
T7-F9254-cRNA	TAATACGACTCACTATAGGacatgctgaacggagtctccaGTTTTAGTACTCTGGAAAC
Reverse	CTATAGTTCTAGAGGCTCGAGAAAAATCTCGCCAACAAGTTG
tyr seqF	ATGACGGCTACTACATGGTGC
tyr seqR	TGTCTAGATGTGGTCGGTGAG
pax6.1 seqF	CAGCTTGGTGGCGTGTTTGTTA
pax6.1 seqR	GTCACATGCACCTTCCCTCCTC
oca2 DF	TGCAAACAGGATCGAGGGTT
oca2 DR	CTGCTTTACTTCGGTTTGGTGT

F: forward direction; R: reverse direction; tRNA: transfer RNA; gRNA: guide RNA; cRNA: CRISPR-derived RNA. Single-guide RNA (sgRNA) sequences are underlined.

et al., 2022), the target fragment in-tRNA was amplified by PCR using primers in-tRNA F/in-tRNA R (Table 1, Fig. 1d), and then inserted into the pCS2+tRNA-mSatyr-sgRNA plasmid digested by *Xho*I (Fig. S2). The PCR programs were set as 94 °C for 3 min, followed by 35 cycles of 94 °C for 25 s, 62 °C for 30 s, and 72 °C for 15 s, and then 72 °C for 3 min. The recombinant plasmid pCS2+tRNA-mSatyr-sgRNA+tRNA was also sequenced for confirmation (Fig. 1d, Seq. S4).

Based on the plasmid pC322mSaCas9, a tRNA-sgRNA-tRNA sequence was inserted between the SaCas9 and SV40 polyA to form plasmid pC322mSaCas9+tRNA-tyr sgRNA+tRNA and synthesized by GenScript Biotech Corp. (Seq. S5). The cassette of mSaCas9+tRNA-tyr sgRNA+tRNA-SV40 polyA was controlled by cytomegalovirus (CMV) and T7 promoters, in which the mRNA of Cas9 and sgRNA can be transcribed from the CMV or T7 promoter.

2.3 mRNA and sgRNA synthesis

The medaka *tyr* sequence (Gene ID: 100049427), *pax6.1* sequence (Gene ID: 100049356), and *oca2* sequence (Gene ID: 100049406) were retrieved from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov) (Chen et al., 2017; Fang et al., 2018; Pan et al., 2023a, 2023b). The spacers were designed using the CCTop (https://crispr.cos.uni-heidelberg.de) and CRISPOR websites (http://crispor.tefor.net). The PAM sequences of R4414, R4445, R4468, F4553, R14069, R14135, F9011, and F9254 were 5'-NNGRRT-3', and those of NR4443 and NR4475 were 5'-NNNNNN-3' (Table 1).

The plasmid pC322mSaCas9 was digested by *Bbs*I as a template for mSaCas9 mRNA, and the mRNA was transcribed using the mMESSAGE mMACHINE T7 transcription kit (Thermo Fisher Scientific) (Fig. S3). The target fragment T7-sgRNA was amplified by PCR using primers T7-gRNA-cRNA/Reverse (Table 1, Fig. 1b), and sgRNAs were transcribed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific) (Seq. S6). The plasmids pCS2+tRNA-mSatyr-sgRNA and pCS2+tRNA-mSatyr-sgRNA+tRNA were digested by *Not*I as templates for SP6-sgRNAs, and sgRNAs were transcribed using TranscripAid SP6 High Yield Transcription Kits (Thermo Fisher Scientific) (Fig. S4).

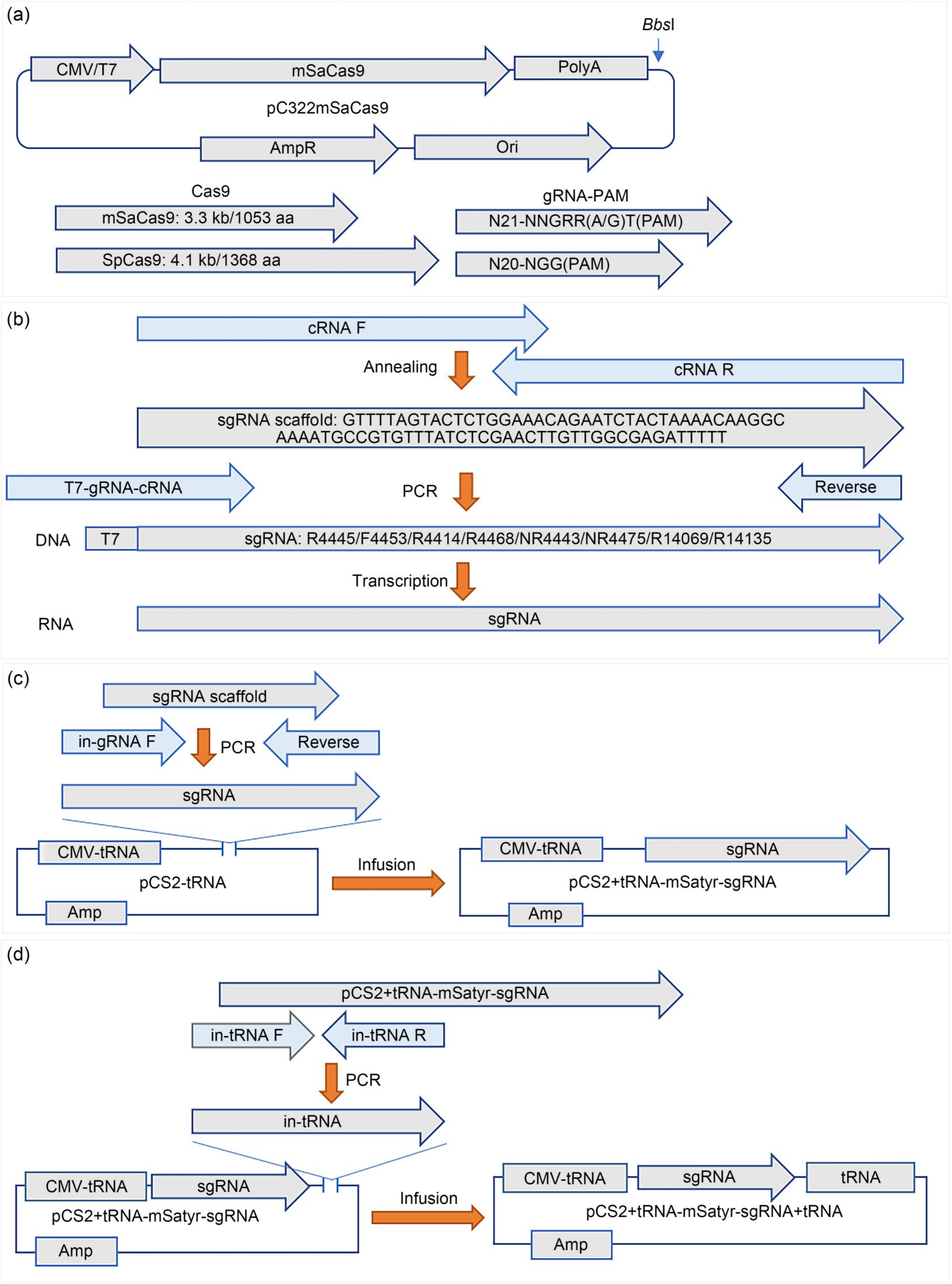

Fig. 1 Syntheses of mSaCas9 DNA, single-guide RNA (sgRNA) scaffold, and sgRNA. (a) Map of *Staphylococcus aureus* Cas9 (SaCas9) expression vector. SaCas9 uses NNGR(A/T)RT as the protospacer-adjacent motif (PAM) sequence, and its length is smaller than that of *Streptococcus pyogenes* Cas9 (SpCas9). (b) Primers were designed and annealed to synthesize mSaCas9 sgRNA scaffold, and spacers carrying the T7 promoter were synthesized using mSaCas9 sgRNA scaffold as a template. (c) Construction of the transfer RNA (tRNA)-sgRNA expression vector. The target fragment sgRNA was ligated into the linearized vector pCS2-tRNA by infusion to obtain a recombinant plasmid pCS2+tRNA-mSatyr-sgRNA including a fragment of tRNA and sgRNA. (d) Construction of the tRNA-sgRNA-tRNA expression vector. The target fragment in-RNA was ligated into the linearized vector pCS2+tRNA-mSatyr-sgRNA by infusion to obtain a recombinant plasmid pCS2+tRNA-mSatyr-sgRNA+tRNA including a fragment of two tRNAs and one sgRNA. CMV: cytomegalovirus; PCR: polymerase chain reaction.

The plasmid pC322mSaCas9+tRNA-tyr sgRNA+tRNA was digested by *Eco*RI as a template for T7-SaCas9-sgRNA, and mRNA-sgRNA was transcribed using the mMESSAGE mMACHINE T7 transcription kit (Thermo Fisher Scientific) (Fig. S5).

2.4 Medaka embryo microinjection, T7 endonuclease (T7EI) assays, and sequencing

A mixture containing sgRNA or plasmid, Cas9 mRNA, and 0.05% (0.5 g/L) phenol red was injected into one-cell-stage embryos (Pan et al., 2022). The specific concentrations are shown in Table 2.

The injected embryos were cultured in embryonic rearing medium and imaged with a Leica M205 FA stereomicroscope (Leica, Germany). Heterozygous mutant embryos with a significant phenotype were collected for genotyping. Each pool of embryos was lysed with cell lysis buffer (10 mmol/L Tris-HCl (pH 8.0), 75 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 0.5% (5 g/L) sodium dodecyl sulfate (SDS)) and proteinase K (100 μg/mL) at 65 °C for 3 h. Proteins and other impurities were removed by adding NH_4Ac (7.5 mol/L), and then the DNA was precipitated with isopropanol and resuspended in Tris-EDTA (TE) buffer solution (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) (Pan et al., 2022). The target fragments were amplified by PCR using primers tyr seqF/tyr seqR, oca2 DF/oca2 DR, or pax6.1 seqF/pax6.1 seqR (Table 1). The PCR programs for tyr seqF/tyr seqR were set as 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and then 72 °C for 5 min. The PCR programs for oca2 DF/oca2 DR and pax6.1 seqF/pax6.1 seqR were set as 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 50 s, and then 72 °C for 5 min. The PCR products with a mismatch site were digested by T7 endonuclease I (EN303-01; Vazyme, China) to detect mutation, and then cloned into pMD18-T (D103A; TaKaRa, Kyoto, Japan) for single-colony sequencing, and the sequence alignment was performed by SnapGene (https://www.snapgene.com).

2.5 Detection of the genetic inheritance of the mutant

The mutant embryos of F0 were raised to adult fish and were selfed, or hybridized with the *tyr* homozygous mutant (*tyr*$^{-/-}$), which was obtained using the SpCas9 system to knock out the *tyr* gene (Fang et al., 2018). Then, the existence of hypopigmentation in hybrid embryos was observed in both strains, SaCas9 *tyr*$^{-/-}$ and SaCas9/SpCas9 *tyr*$^{-/-}$. The homozygous mutants of strains were confirmed by sequencing.

3 Results

3.1 Preparation of SaCas9 mRNA, sgRNA, and vectors

The plasmid pC322mSaCas9 was 8266-bp long (Fig. S3), and the length of mSaCas9 mRNA obtained by in vitro transcription was 3361 nucleotides (nt) after being linearized by *Bbs*I. The SaCas9 is driven by the CMV promoter for eukaryotic expression in cells or

Table 2 Contents of injections used in the experiments

Group	Injection content (gene; DNA/RNA)	Injection number	Survival number	Mutation number	Mutation rate (%)
WT	NA	0	192	0	0
Group 1	R4445+F4553 sgRNA (*tyr*; RNA)	194	135	56	41.5
Group 2	R14069+R14135 sgRNA (*pax6.1*; RNA)	81	73	23	31.5
Group 3	R4414+R4468 sgRNA (*tyr*; RNA)	92	51	29	56.9
Group 4	NR4443+NR4475 sgRNA (*tyr*; RNA)	50	39	0	0
Group 5	SP6-pCS2+tRNA-mSatyr-sgRNA (*tyr*; RNA)	30	23	7	30.4
Group 6	SP6-pCS2+tRNA-mSatyr-sgRNA+tRNA (*tyr*; RNA)	124	100	43	43.0
Group 7	pCS2+tRNA-tyr R4445 (*tyr*; DNA)	239	129	9	7.0
Group 8	pCS2+tRNA-tyr-sgRNA+tRNA (*tyr*; DNA)	216	152	12	7.9
Group 9	F9011+F9254 (*oca2*; RNA)	50	41	40	97.6
Group 10	mSaCas9-tRNA-tyr sgRNA-tRNA (*tyr*; RNA)	52	50	49	98.0
Group 11	mSaCas9-tRNA-tyr sgRNA-tRNA+tyr sgRNA (*tyr*; RNA)	54	51	44	86.3

WT: wild type; NA: not applicable; sgRNA: single-guide RNA; tRNA: transfer RNA; *tyr*: tyrosinase; *oca2*: oculocutaneous albinism II; *pax6.1*: paired box 6.1.

embryos, and the SaCas9 mRNA can be transcribed by the T7 promoter in vitro (Fig. 1a). The sgRNA scaffold template was annealed by oligo pairs, and the DNA of the sgRNA was obtained by PCR using forward and reverse primers. The forward primer contained the T7-spacer and a part sequence of the sgRNA scaffold (Fig. 1b).

The sgRNA expression vector was constructed by infusing the sgRNA fragment with the pCS2-CMV-tRNA vector (Fig. 1c), in which the RNA can be transcribed by the SP6 promoter in the pCS2-CMV-tRNA-sgRNA vector. Moreover, additional tRNA was added to the vector to form the pCS2-CMV-tRNA-sgRNA-tRNA, and the sgRNA was protected by dual tRNAs, front and back (Fig. 1d). All the vectors were confirmed by sequencing.

3.2 Knockout of the medaka *tyr*, *oca2*, and *pax6.1* genes using the msSaCas9 system

The medaka *tyr* genomic DNA contains six exons. The sgRNAs of R4445/F4553 were designed in the fifth coding exon (Fig. 2a). Three days post-injection (dpi) of embryos with the Cas9 and sgRNAs, the absence of

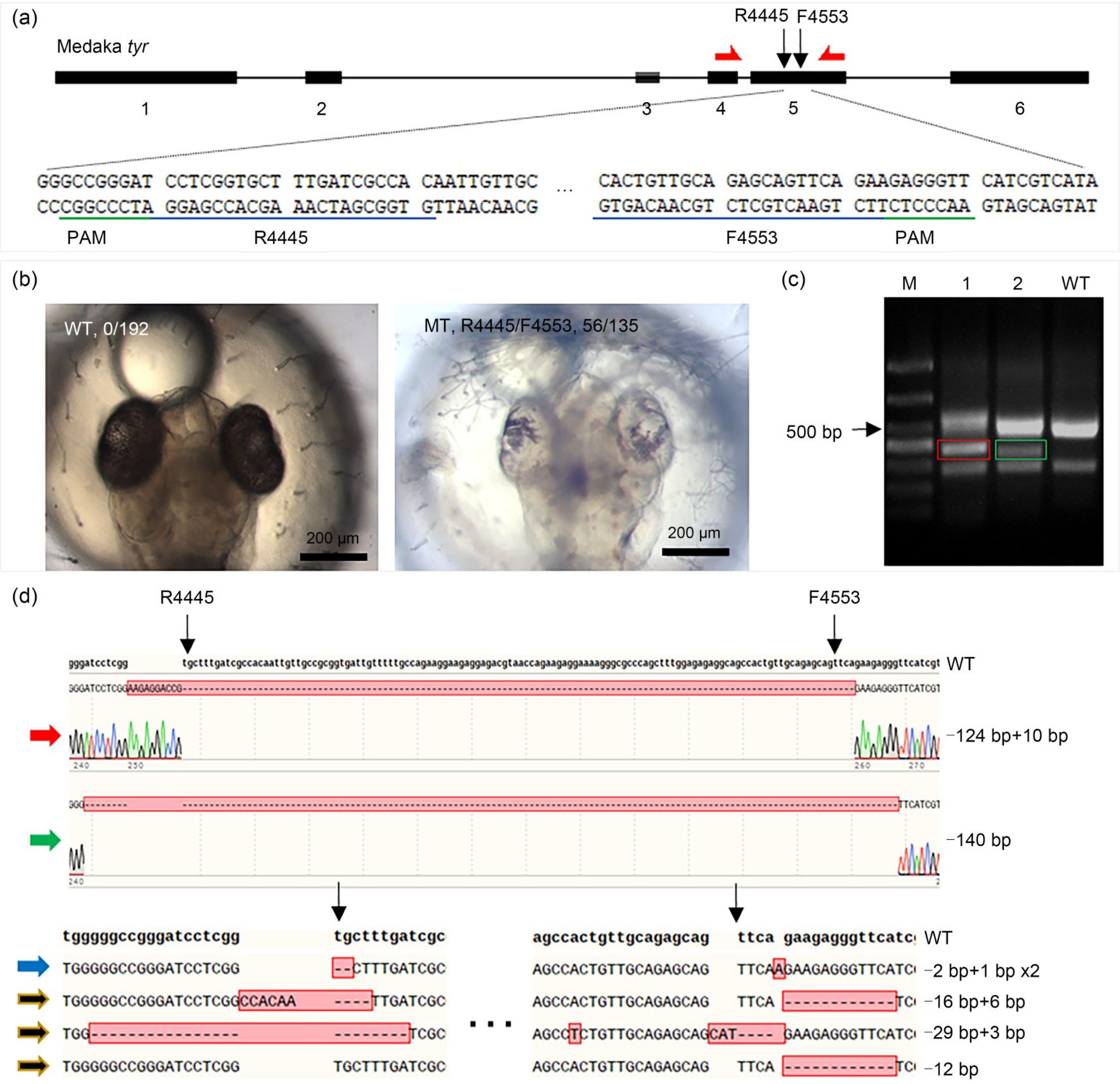

Fig. 2 Analyses of R4445 and F4553 knockout in tyrosinase (*tyr*) gene. (a) The sequences of R4445 and F4553 are underlined in blue, while protospacer-adjacent motif (PAM) sequences are underlined in green. (b) A significant loss of pigmentation was observed in the mutation group (MT, 56/135) compared to wild type (WT, 0/192). (c) The PCR products from heterozygous mutant embryos (1, 2) and wild-type embryos (WT) were treated with T7 endonuclease (T7EI). (d) Monoclonal sequencing of different bands. There were several base mutations in the single or dual single-guide RNA (sgRNA) knockout sites. PCR: polymerase chain reaction; M: marker.

pigment from the eye (56/135) was observed (Fig. 2b). This was not observed in the wild-type (WT) embryos (*n*=192). Moreover, the PCR and sequencing results demonstrated that both sgRNAs were functional, which was confirmed by sequencing in both sites (Figs. 2c and 2d). In addition, both sequence mutations were found near two sgRNAs except for the deletion of more than 120 bp between the two sgRNAs (Fig. 2d). Notably, we also observed that the different mutation sequences occurred in the same embryo when we analyzed single embryos by sequencing (Fig. 2d, black arrows), meaning that gene editing is a dynamic process.

To investigate the editing of more genes, we also tested the *oac2* gene, which is another important gene for pigmentation during eye development. Two sgRNAs, F9011 and F9254, were designed in exon 5 or exon 7 accordingly. Similarly, the pigmentation was partially lost in the injected embryos (Figs. 3a and 3b; 40/41), and the mutations were confirmed in both sgRNAs (Fig. 3c).

The *pax6.1* gene, another master gene for eye development in medaka, was also tested. The sgRNAs were designed in exon 6. Similarly, ocular malformation of the eye (Figs. 4a and 4b; 23/73) was observed in the *pax6.1* sgRNA group at 5 dpi, and the mutations were confirmed by sequencing in the sgRNA (Figs. 4c and 4d; R14135). Therefore, the SaCas9 system can be used effectively and easily to edit the medaka genome.

3.3 Necessity of PAM for the mSaCas9 system

To test whether the PAM sequence of 5'-NNGRRT-3' is necessary for gene editing, we also used an alternative PAM sequence of 5'-NNNNNN-3' (NR4443/NR4475) (Figs. 5a–5c). We also extended the spacer from 21 to 24 nt in the control group (R4414/R4468,

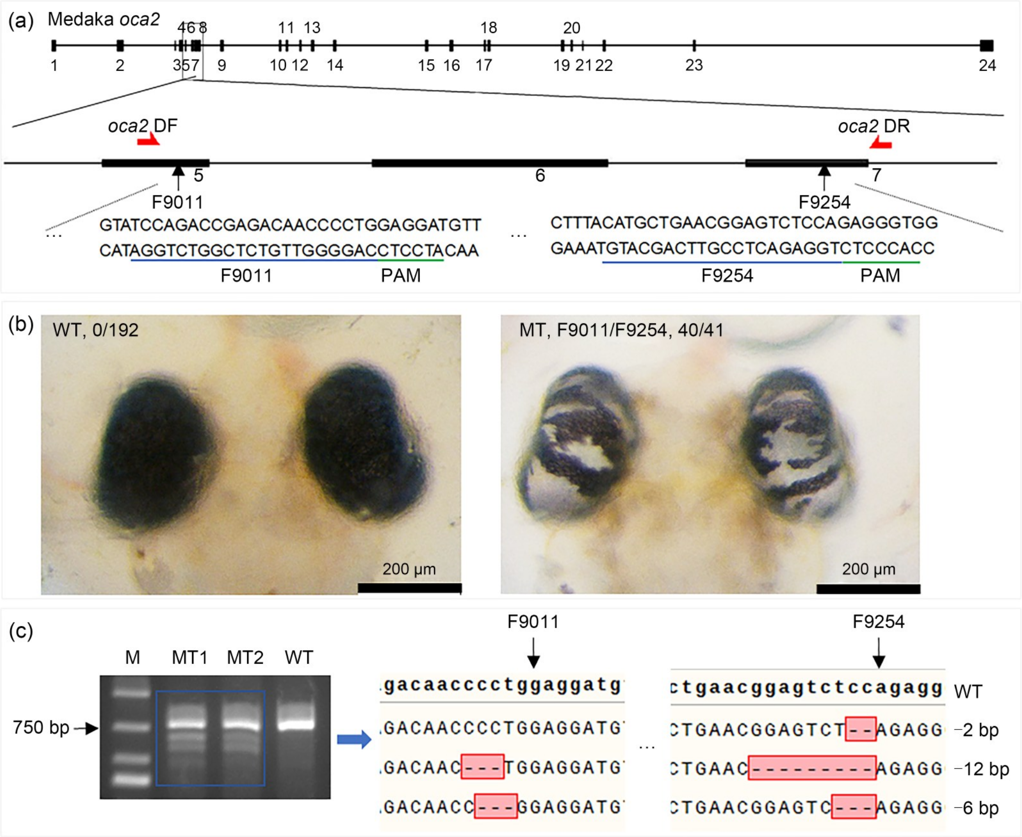

Fig. 3 Analyses of F9011 and F9254 knockout in oculocutaneous albinism II (*oca2*) gene. (a) The sequences of F9011 and F9254 are underlined in blue, while protospacer-adjacent motif (PAM) sequences are underlined in green. (b) Compared with wild-type embryos (WT, 0/192), the heterozygous mutations showed a significant pigment deletion in the mutation group (MT, 40/41). (c) Monoclonal sequencing of PCR fragments. The PCR products from mutant (MT1/MT2) and WT embryos were treated with T7 endonuclease (T7EI). Different types of mutations were found in the same embryo. PCR: polymerase chain reaction; M: marker.

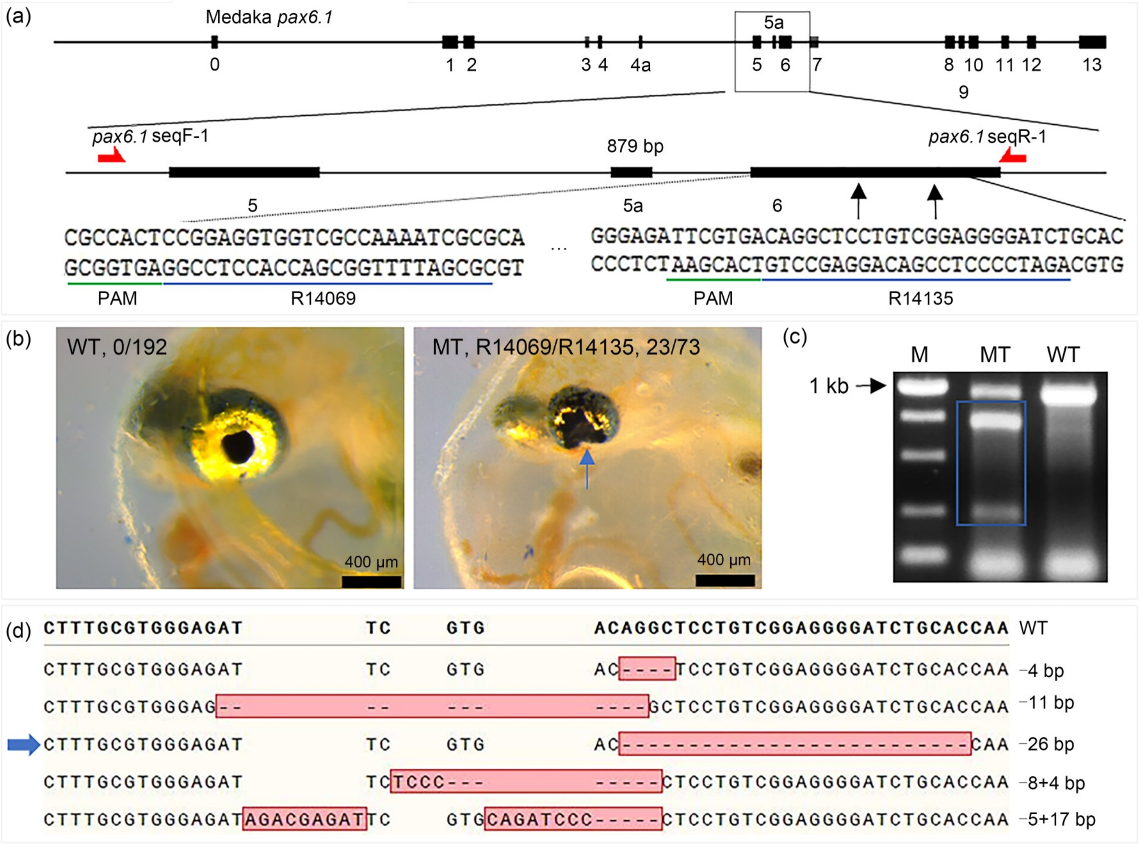

Fig. 4 Analyses of R14069 and R14135 knockout results in paired box 6.1 (*pax6.1*) gene. (a) The sequences of R14069 and R14135 are underlined in blue, while protospacer-adjacent motif (PAM) sequences are underlined in green. (b) Compared with wild-type embryos (WT, 0/192), the heterozygous mutant embryos (MT, 23/73) showed significant ocular malformation. (c) The PCR products from MT and WT embryos were treated with T7 endonuclease (T7EI). (d) Monoclonal sequencing of PCR fragments. There were several types of mutations only in the R14135 knockout site. PCR: polymerase chain reaction; M: marker.

24 nt) (Figs. 5a–5c). While no phenotype was detected in the NR group (NR4443/NR4475, 0/39), the control group that was injected with R4414/R4468 (24 nt, 29/51) showed a considerable difference in the eye (Fig. 5b). Moreover, the mutation sequences were confirmed in the mutant group but not in the NR group (Fig. 5c). Therefore, the PAM sequence 5'-NNGRRT-3' is essential for gene editing, and the extended 24-nt spacer is also acceptable, as tested for the three genes above.

3.4 Mediation of functional sgRNA by tRNA in the SaCas9 system

Generation of the functional sgRNA is an essential part of gene editing, which is always a challenge in divergent species. To test whether the sgRNA can function in vivo or in vitro by linking to the tRNA, which is an ideally and systematically self-processed cellular component, we also injected the tRNA-sgRNA mixed with Cas9. As in the embryos injected with the sgRNA mixture (Fig. 2a), the absence of pigment from the eye was observed in the tRNA-sgRNA (Figs. 6a and 6b; 7/23) and tRNA-sgRNA-tRNA embryos (Fig. 6b; 43/100), indicating that tRNA mediates functional sgRNA in medaka embryos by RNA injection. The absence of pigment from the eye was also observed in the plasmid group (Fig. 6b; 12/152), indicating that the tRNA-sgRNA-tRNA system is also functional following DNA injection. Sequencing also confirmed the mutation (Fig. 6c).

3.5 All-in-one promoter system to mediate gene editing by tRNA

To test whether the tRNA could also mediate the Cas9 protein and sgRNA stimulation, we inserted the tRNA between the SaCas9 and sgRNA, in which only

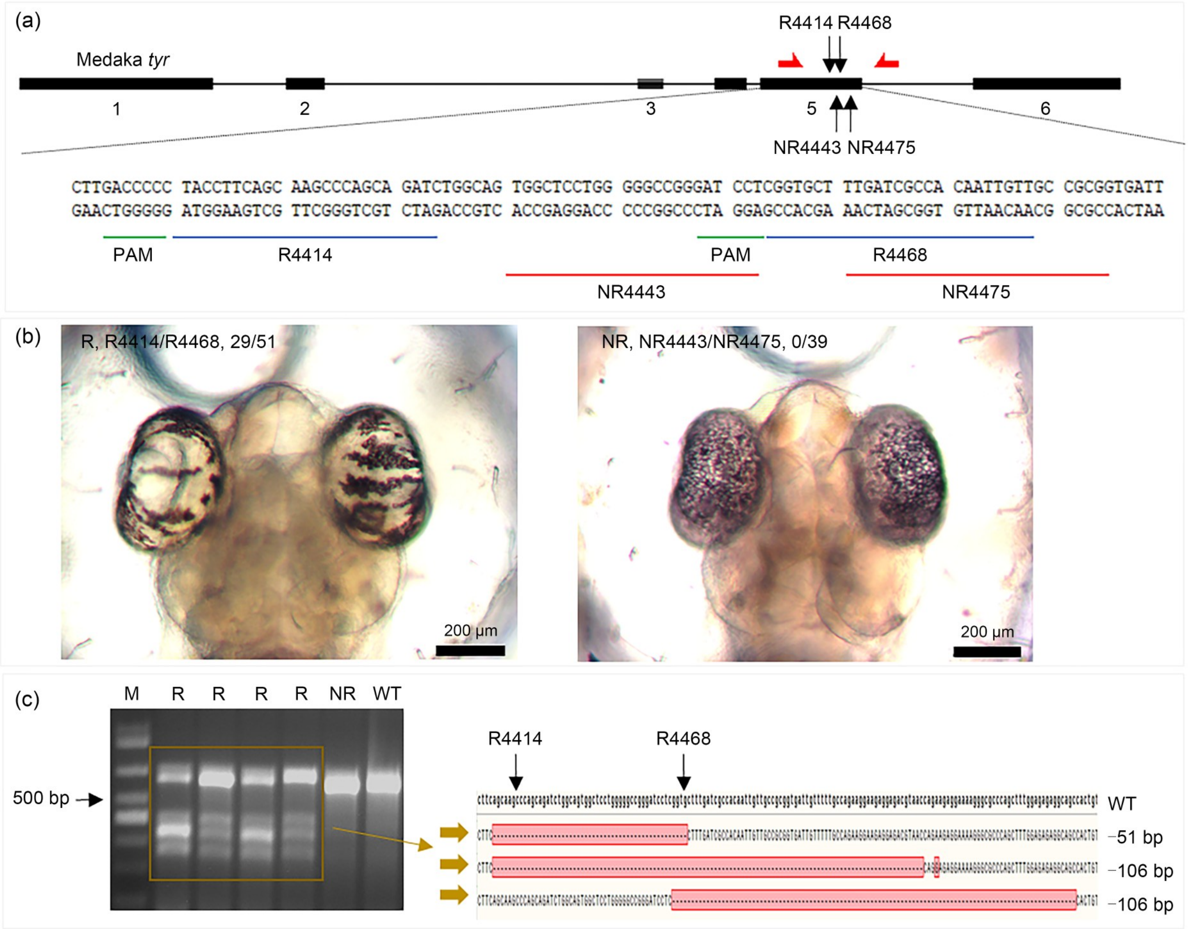

Fig. 5 Analyses of R4414+R4468 and NR4443+NR4475 knockout results in tyrosinase (*tyr*) gene. (a). The sequences of R4414 and R4468 are underlined in blue, and those of NR4443 and NR4475 are underlined in red. The protospacer-adjacent motif (PAM) sequences are underlined in green. (b) The heterozygous mutants showed significant pigment deletion in the R4414+R4468 group (R, 29/51) but not in the NR4443+NR4475 group (NR, 0/39). (c) Monoclonal sequencing of PCR products. The PCR products from mutation (R), NR, and wild type (WT) groups were treated with T7 endonuclease (T7EI). There were several base mutations in the single-guide RNA (sgRNA) knockout sites in the R group but not in the NR or WT groups. PCR: polymerase chain reaction; M: marker.

the promoter CMV/T7 controls the expression of Cas9 and sgRNA together (Figs. 7a and 7b). The RNAs were transcribed by T7 RNA polymerase and injected into embryos with or without the verified sgRNA (R4445). As expected, the absence of pigment from the eye was also observed in both the mSaCas9-tRNA-sgRNA-tRNA embryos (mRNA without sgRNA, 49/50) and mSaCas9-tRNA-sgRNA-tRNA+sgRNA embryos (mRNA with gRNA, 44/51) (Fig. 7b), and the mutations were also confirmed by sequencing (Fig. 7c). This indicates that tRNA mediates functional sgRNA and SaCas9 in medaka embryos following RNA injection. Therefore, the tRNA mediates the sgRNA function in the embryo following either RNA or DNA injection, either of which could potentially be used in the fish cells.

3.6 Generation of the heritable mutants

After we observed the loss of pigmentation, the mutated embryos were raised to adult fish and homozygotes were produced following standard procedures. The homozygotes produced by SpCas9 were also used to hybridize with homozygotes produced by SaCas9. Compared with the black eye color of WT medaka, the eyes were red color in SpCas9 and SaCas9 strains, in both males and females (Figs. 8a–8f). Consequently, hybridized albinos can also be produced, showing the red color in the eye and mutated sequence in different strains (Fig. 8g). In addition, all mutant strains were maintained stably for more than eight generations in the lab.

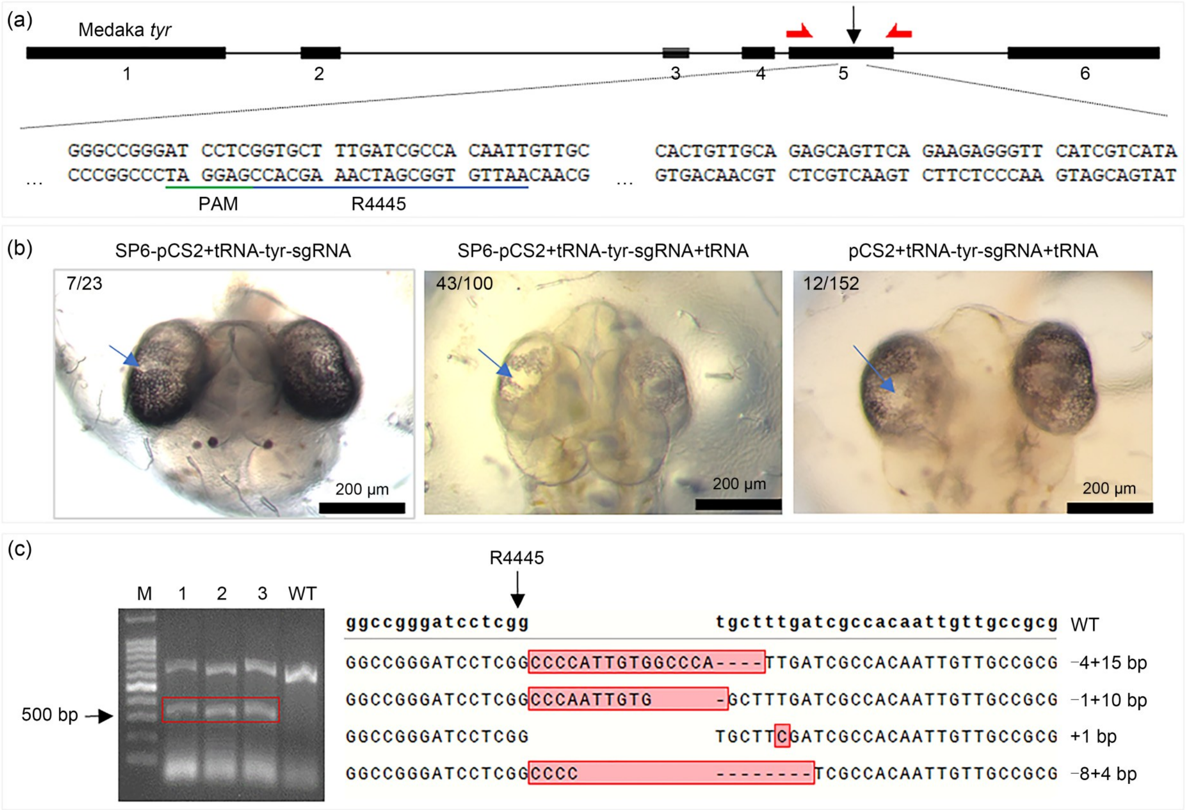

Fig. 6 Analyses of transfer RNA (tRNA)-single-guide RNA (sgRNA) in RNA or DNA content in tyr-sgRNA-tRNA knockout results. (a) The sequence of R4445 is underlined in blue, while that of the protospacer-adjacent motif (PAM) is underlined in green. (b) The heterozygous mutants showed a significant pigment deletion in SP6-pCS2+tRNA-tyr-sgRNA (7/23), SP6-pCS2+tRNA-tyr-sgRNA-tRNA (43/100), and pCS2+tRNA-mSatyr-sgRNA+tRNA DNA groups (12/152). (c) Monoclonal sequencing of PCR products. The PCR products from mutants (1–3) and wild type (WT) were treated with T7 endonuclease (T7EI). There were several types of mutations in the R4445 knockout site in different groups. *tyr*: tyrosinase; PCR: polymerase chain reaction; M: marker.

4 Discussion

The gene-editing system is the most powerful tool developed during the last decade to analyze gene function and generate specific mutants. Compared to the well-established SpCas9 system that is used in different species including mammals, teleostean fish, and bacteria, in this study we extended the SaCas9 system and examined editing efficiency in medaka fish.

Firstly, the smaller SaCas9 is functional in the medaka genome. SaCas9 (3.3 kb) is almost 25% smaller than SpCas9 (nearly 4.3 kb). This facilitates cloning, transcription, and transfection for different purposes. Researchers have demonstrated the ability to use the SaCas9 system to edit genomes efficiently and have applied it to mammals using AAV viruses for gene editing in different models (Zhang et al., 2016; Li et al., 2018). In this study, we successfully used a codon-optimized SaCas9 system for gene editing of medaka. Moreover, the RNA transcription or DNA vector of a particular gene sgRNA can be obtained easily by simple PCR or one-step cloning.

Secondly, the SaCas9 can mediate gene editing in both sgRNAs. By sequencing, we found that gene editing occurred in single sgRNA or both sgRNAs. Most importantly, there were few base pairs of mutations near both sgRNAs, except for the deletion of a whole fragment between the two sgRNAs, meaning that the mutations had occurred simultaneously in some cases. We also found that different mutation types can be detected in a single embryo, indicating that gene editing can occur in different cells during embryonic cleavage, possibly due to the high concentration of injected Cas9 and sgRNAs. For example, gene editing happened in one,

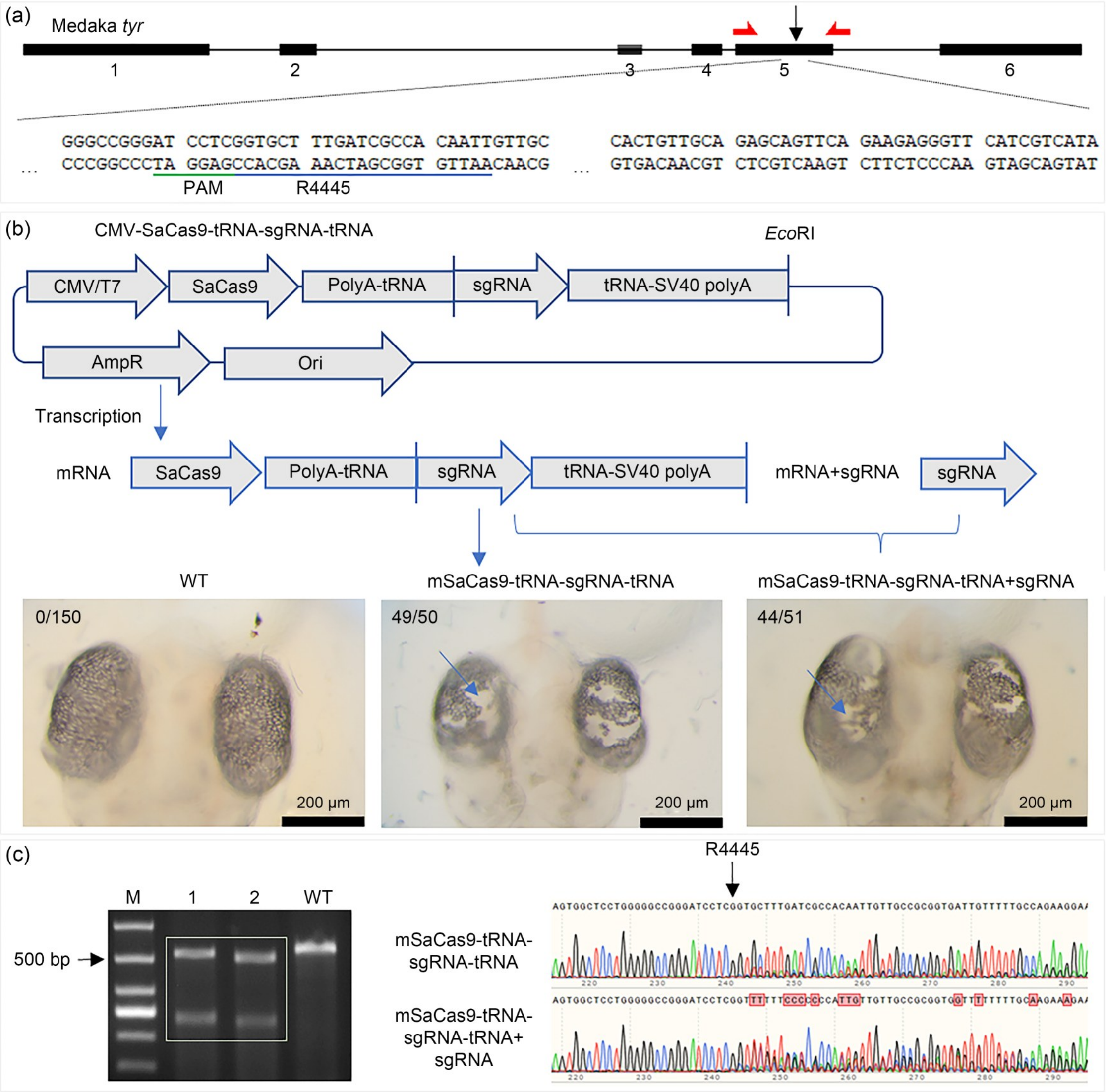

Fig. 7 mSaCas9-transfer RNA (tRNA)-single-guide RNA (sgRNA) in tyrosinase (*tyr*) gene knockout results. (a) The sequence of R4445 is underlined in blue, while that of the protospacer-adjacent motif (PAM) is underlined in green. (b) An illustration of the all-in-one vector cytomegalovirus (CMV)-SaCas9-tRNA-sgRNA-tRNA. The heterozygous mutants showed a significant pigment deletion in the mSaCas9-tRNA-tyr sgRNA-tRNA (49/50) and mSaCas9-tRNA-tyr sgRNA-tRNA+tyr sgRNA groups (44/51). (c) The PCR products from mutants (1, 2) and wild type (WT) were treated with T7 endonuclease (T7EI), and multiple peaks were detected in the sequencing of the PCR products. PCR: polymerase chain reaction; M: marker.

two, or four cells, resulting in the different mutant types. Consequently, different mutants can be obtained from only a few (such as ten to twenty) injected embryos if the sgRNA is suitable, which reduces the investment needed to screen the large number of mutants in subsequent steps.

Thirdly, the PAM sequence 5'-NNGRRT-3' is also important for gene-editing efficiency, as confirmed in this study (Friedland et al., 2015; Ran et al., 2015; Brooks and Gaj, 2018; Xie et al., 2018). No mutation could be obtained by changing the PAM sequence of 5'-NNGRRT-3' to a random sequence of 5'-NNNNNN-3'. In addition, extending the spacer from 21 to 24 nt did not affect its function. Moreover, the sgRNA was still functional when either was placed behind a tRNA or inserted between two tRNAs, which is important for enabling vector cloning of sgRNA expression by conventional promoters such as the CMV promoter (Pan et al., 2022). Most important, the transcribed RNA from one promoter, such as T7, is also functional for

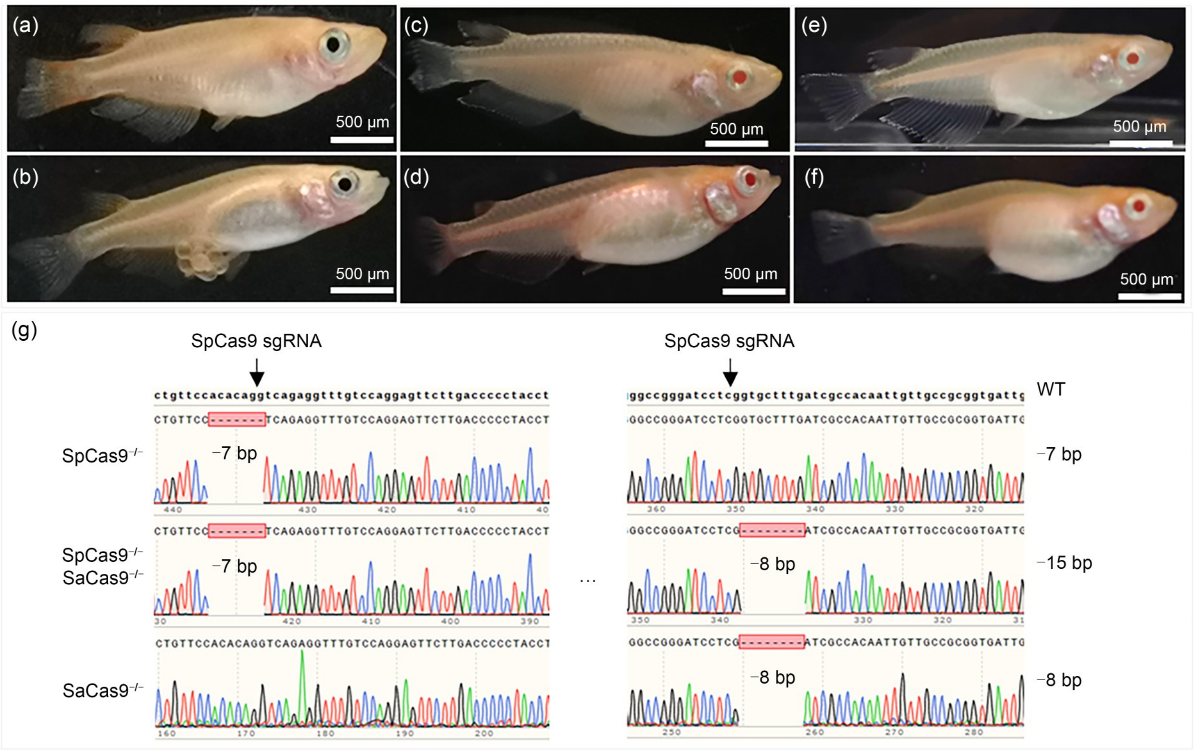

Fig. 8 Adult fish of the tyrosinase (*tyr*) mutant generated by the *Staphylococcus aureus* Cas9 (SaCas9) or *Streptococcus pyogenes* Cas9 (SpCas9) system. (a) Wild-type (WT) male medaka (♂). (b) WT female medaka (♀). (c) The male homozygote of the SaCas9 knockout (♂). (d) The female homozygote of the SaCas9 knockout (♀). (e) The male homozygote of the SpCas9 knockout (♂). (f) The female homozygote of the SpCas9 knockout (♀). (g) Monoclonal sequencing of PCR products in WT and homozygous medaka. There were mutations in different single-guide RNA (sgRNA) regions in the SpCas9 and SaCas9 strains. PCR: polymerase chain reaction.

SaCas9 translation and sgRNA processing, and the two parts are functional, as verified in this study. Therefore, the combination of SaCas9/tRNA-sgRNA is suitable for gene editing of medaka embryos.

Lastly, stable and heritable mutants can be readily generated. We obtained *tyr*$^{-/-}$ albino strains using SpCas9 (Fang et al., 2018), SaCas9, or a hybrid of both systems, suggesting that SaCas9 may be an easy and acceptable system to generate other strains with heritable mutations.

Taken together, the SaCas9 editing system has been optimized and verified in medaka to generate mutants and could also be applied to other fish cell lines or other teleosts. Ideally, a minimal cassette for SaCas9-sgRNA expression will be desired for robot gene mutation in the future.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

Yuewen JIANG performed the experiment, analyzed experimental data, and wrote the original draft. Qihua PAN, Zhi WANG, Ke LU, and Bilin XIA participated in part of the experiment and edited the manuscript. Tiansheng CHEN acquired research funding, designed and supervised the experiment, analyzed experimental data, and wrote and edited the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yuewen JIANG, Qihua PAN, Zhi WANG, Ke LU, Bilin XIA, and Tiansheng CHEN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. All procedures complied with the protocols approved by the Animal Care and Use Committee of Jimei University, Xiamen, China (No. JMU202203009).

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Supplementary information

Seqs. S1–S6; Figs. S1–S5