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ORIGINAL ARTICLE

Production of Homozygous Transgenic Rainbow Trout with Enhanced Disease Resistance

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Abstract Previous studies conducted in our laboratory showed that transgenic medaka expressing cecropin B transgenes exhibited resistant characteristic to fish bacterial pathogens, *Pseudomonas fluorescens* and *Vibrio anguillarum*. To confirm whether antimicrobial peptide gene will also exhibit anti-bacterial and anti-viral characteristics in aquaculture important fish species, we produced transgenic rainbow trout expressing cecropin P1 or a synthetic cecropin B analog, CF-17, transgene by sperm-mediated gene transfer method. About 30 % of fish recovered from electroporation were shown to carry the transgene as determined by polymerase chain reaction (PCR) amplification assay. Positive P_1 transgenic fish were crossed to non-transgenic fish to establish F_1 transgenic founder families, and subsequently generating F_2, and F_3 progeny. Expression of cecropin P1 and CF-17 transgenes was detected in transgenic fish by reverse transcription (RT)-PCR analysis. The distribution of body sizes among F_1 transgenic fish were not significantly different from those of non-transgenic fish. Results of challenge studies revealed that many families of F_2 and F_3 transgenic fish exhibited resistance to infection by *Aeromonas salmonicida* and infectious hematopoietic necrosis virus (IHNV). All-male homozygous cecropin P1 transgenic families were produced by androgenesis from sperm of F_3 heterozygous transgenic fish in one generation. The resistant characteristic to *A. salmonicida* was confirmed in progeny derived from the outcross of all-male fish to non-transgenic females. Results of our current studies confirmed the possibility of producing disease-resistant homozygous rainbow trout strains by transgenesis of cecropin P1 or CF-17 gene and followed by androgenesis.

Keywords Cecropin P1 · Antimicrobial peptide · Transgenic rainbow trout · Disease resistant · Sperm-mediated gene transfer · Electroporation

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Introduction

Cecropins, a family of small molecular weight basic peptides with bactericidal activity, also known as antimicrobial peptide (AMP), were first discovered in the hemolymph of diapausing cecropia (*Hyalophora cecropia*) pupae following inoculation with bacterial debris (Hultmark et al. 1980; Boman and Hultmark 1987). Since then many cecropin-like antimicrobial peptides have been identified and characterized from a wide variety of organisms including nematode (Pillai et al. 2005), invertebrates, vertebrates (for review, see Bulet et al. 2004) and plants (Broekaert et al. 1995). Cecropins are first translated as prepropeptides containing 62 to 64 amino acid residues, and then processed intracellularly into mature peptides of 35 to 37 amino acid residues prior to secretion into the circulation

(Boman et al. 1991; Boman 1995). Due to their unique structural features, cecropins and cecropin-like peptides can be readily incorporated into the plasma membranes of bacteria resulting in the formation of pores on the plasma membrane and leading to the inevitable death of prokaryotic and eukaryotic pathogens (Bechinger 1997). Many cecropin analogs have been designed and synthesized, and these peptides are as effective as or even more potent than their native molecules against plant and animal bacterial pathogens and protozoa (Kadonon-Okuda et al. 1995; Merrifield et al. 1995; Rodriguez et al. 1995; Vunnam et al. 1995). Genes (cDNAs and genomic sequences) encoding cecropins and their analogues have been cloned from insects (van Hofsten et al. 1985; Tian et al. 2010), nematode (Pillai et al. 2005), shrimp (Destoumieux et al. 1997; Chiou et al. 2005), and vertebrates (Syvitski et al. 2005). By the use of gene transfer technology, genes of cecropins and their analogues have been used to produce transgenic plants (Jaynes et al. 1987; Hassan et al. 1993; Jia et al. 1993; Huang et al. 1997; Osusky et al. 2000) with increased resistance to infection by bacterial or fungal pathogens.

Fish diseases resulting from infection by bacterial, viral and parasitic pathogens are one of the most severe bottlenecks in aquaculture (Inglis and Hendrie 1993; Thune et al. 1993). For the past few decades, efforts to control infectious diseases in commercially important fish species have primarily depended on employing antibiotics, developing suitable vaccines and selecting fish strains with robust resistance to infectious pathogens. Although control of fish diseases by using antibiotics is effective, the number of antibiotics approved for treating diseased fish infected by bacterial pathogens is limited (Post 1987). Furthermore, the increasing number of antibiotic-resistant pathogenic microorganisms in the aquatic environment challenges the strategy of using antibiotics to control fish diseases (Fjalestad et al. 1993). While effective vaccines have been developed for several important fish pathogens over the past many years, the current vaccination practice is expensive, laborious and time consuming (Fjalestad et al. 1993; Ganz 1999). The advantage of using vaccination technology is often out-weighed by its labor-intensiveness. Genetic selection based on traditional cross-breeding techniques is time-consuming and the outcome is frequently unpredictable, and sometimes disappointing due to lacking the desired genetic traits. Therefore, more effective approaches for controlling fish disease in aquaculture are highly desirable.

In vitro studies previously conducted in our laboratory have shown that antimicrobial peptides such as cecropin B, cecropin P1, pleurocidin and CF-17 peptide (a synthetic cecropin B analogue) exhibited bactericidal activity to fish bacterial pathogens such as *Pseudomonas flourescens, Aeromonas hydrophila* and *Vibrio anguillarum* (Chiou et al. 2002; Sarmasik and Chen 2003; Chiou et al. 2006). Furthermore, Chiou et al. (2002) showed in in vitro studies that cecropin P1 and CF-17 peptide effectively inhibited the replication of fish viruses such as infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV) and infectious pancreatic necrosis virus (IPNV). Additionally, Jia et al. (2000) demonstrated the enhanced resistance to bacterial infection in fish which were continuously transfused with a cecropin–melittin hybrid peptide (CEME) and pleurocidin amide, a C-terminally amidated form of the natural flounder peptide. These results suggested that production of disease-resistant fish strain might be achieved by manipulating AMP genes. To test this hypothesis, Sarmasik et al. (2002) introduced transgene constructs containing prepro-cecropin B, procecropin B, mature cecropin B and cecropin P1 into the embryos of medaka by electroporation. The F_2 transgenic medaka was subjected to bacterial challenges at an LD_{50} dose with *Pseudomonas fluorescens* and *V. anguillarum*, respectively. The resulting relative percent survival (RPS) of the tested transgenic F_2 fish ranged from 72 % to 100 % against *P. fluorescens* and 25 % to 75 % against *V. anguillarum*, respectively.

To confirm the feasibility of producing disease-resistant fish strains by manipulating antimicrobial peptide genes in aquaculture fish species, we have transferred cecropin P1 or CF-17 transgenes, under the control of a cytomegalovirus (CMV) promoter, into rainbow trout by sperm-mediated gene transfer method. A total of eight families of cecropin P1 and nine families of CF-17 transgenic rainbow trout were established and bred to all-male homozygous. In this article, we report that the progeny of these transgene-expressing rainbow trout strains exhibited resistance to infection by *Aeromonas salmonicida* and IHNV.

Materials and Methods

Fish Stock and Transgene Constructs

Sperm and eggs of rainbow trout (*Oncorhynchus mykiss*) were obtained from Troutlodge (Sumner, WA, USA) or Roarding River Trout Hatchery (Scio, OR, USA). Fish from embryos to adulthood were reared in tanks with flow through fresh water (12–15 °C) in the John L. Fryer Salmon Disease Laboratory of Oregon State University (Corvallis, OR, USA). Fish were fed to satiety, once a day, with pelleted trout feed (Melick Aqua Feeds, Catawissa, PA, USA).

Cecropin P1 transgene construct was provided by Dunham et al. (2002). Briefly, the coding sequence of mature cecropin P1 cDNA fused with the signal peptide sequence of catfish immunoglobulin heavy chain (Ig) was cloned in frame into an expression vector, pRC/CMV (5.5 kb; Invitrogen, Carlsbad, CA, USA) under the control of a CMV promoter (Fig. 1). CF-17 transgene construct was constructed by replacing sequence of cecropin P1 with a synthetic gene of cecropin analog (Fig. 1).

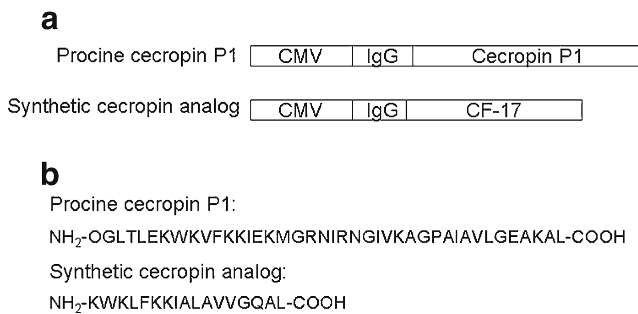

Fig. 1 **a** Structures of transgene constructs used to produce transgenic rainbow trout. **b** Amino acid sequences of porcine cecropin P1 and the synthetic cecropin analogue, CF-17. *CMV* cytomegalovirus promoter region, *IgG* signal peptide sequence of catfish immunoglobulin heavy chain, *cecropin P1* porcine cecropin P1 gene, *CF-17* synthetic cecropin analog

Production of Transgenic Fish

Sperm was collected dry from reproductive mature males and kept at 4 °C until use. Trout sperm can remain un-activated by making one to four dilutions with phosphate buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO4, 0.24 g/l, KH_2PO_4, pH 7.6). Mix 300 μl of sperm (usually ~10^{10} sperm/ml) gently into 600 μl of linearized transgene construct (about 96 μg in PBS) in a cuvette to obtain 10^3 to 10^4 DNA molecules/sperm. The mixture was electroporated in a "Cell-Porator" (BRL, Rockville, MD, USA) under the following condition: resistance, low; capacitance, 1,180 μF; voltage, 300 V; pulses, 2. After electroporation, sperm samples were kept on ice for 10 min to revive, and then added dropwise to 500 eggs in a 1-l container. The eggs were swirled around several times, left at room temperature for 5 min and then activated with water. The fertilized eggs were poured into hatching trays and dead eggs were removed by 48 h after fertilization. The hatched fry were reared to adulthood for genotyping for the presence of the transgene.

Genomic DNA samples were isolated from a small piece of fin tissue collected from the caudal fin at 8 months of age and screened for the presence of transgene sequence by polymerase chain reaction (PCR) amplification and Southern blot hybridization of the PCR products (Chen et al. 1993). The primer sequences for amplification of cecropin P1 and CF-17 transgenes are listed in Table 1. The positive fish were referred to as P_1 transgenic individuals. After reproductive maturation, these fish were crossed with non-transgenic counterparts to produce F_1, F_2 and F_3 heterozygous transgenic fish for disease challenge studies.

Determination of Transgene Expression

Expression of cecropin P1 transgene or CF-17 transgene in the individual F_1, F_2 or F_3 transgenic fish was determined by reverse transcription (RT)-PCR analysis. Total RNA samples were isolated from tissues of liver, muscle and spleen of transgenic individuals using Trizol reagent according to the manufacturer's protocol (Invitrogen). To confirm that RNA samples were free of DNA contamination, RNA samples were used as templates for direct PCR amplification of β-actin sequence without RT. One microgram of total RNA from transgenic or non-transgenic fish was reverse transcribed using SuperScript III reverse transcriptase in a 20-μl reaction volume containing 100 ng oligo-dT, 1 mM dNTP, 5 mM DTT, 1× reaction buffer, 1 unit of RNasin (Promega, Wisconsin, USA), and 1 μl of Superscript III reverse transcriptase according to the manufacture's protocol (Invitrogen). After RT, 1 μl of RT mix was used for the subsequent PCR amplification in a volume of 50 μl containing 200 μM dNTP, 1.5 mM $MgCl_2$, 0.3 μM each gene specific primer and 1.25 units of Taq DNA polymerase (New England Biolaboratories, Massachusetts, USA). The amplification profile contained the following cycles: 1 cycle of 95 °C for 3 min, 40 cycles of 95 °C for 15 s, desired annealing temperature for 15 s, and 72 °C for 30 s. The PCR products were analyzed by electrophoresis on 1.2 % agarose gels.

Histological Examination

Rainbow trout fry were infected with *A. salmonicida* or IHNV-RB, and the morbid fry were collected, euthanized and fixed in 10 % buffered formalin (10 % formalin, 33 mM sodium phosphate monobasic monohydrate, 46 mM sodium phosphate dibasic heptahydrate). The fixed samples were embedded in Paraplast paraffin (Oxford Labware), and sliced sagittally into 6-μm sections. The sections were placed on glass slides and stained with hematoxylin and eosin.

Pathogen Challenge Studies

Single colony of *A. salmonicida* was inoculated into 50 ml of 3 % tryptic soy broth (BD, Franklin Lakes, NJ, USA) containing 1 % NaCl (TSB) and grew at 20 °C overnight. Then, 5 ml of the bacterial cells from the overnight culture was inoculated into 250 ml of fresh TSB the next day and grew for an additional 4 h at 20 °C until reaching mid-log phase. The concentration of bacteria was determined by direct cell counting using a hemocytometer. Fresh IHNV stock was prepared and titer determined following the method described previously (Engelking and Leong 1981), and stored at 4 °C until use.

Three groups each of non-transgenic, F_2 or F_3 heterozygous transgenic fish (30 fish per each, 1–2 g/fish) were used in the challenge studies with *A. salmonicida* at the dose of 5×10^5 colony forming unit (cfu)/ml or IHNV at the dose of 5×10^5 plaque forming unit (pfu)/ml. The challenge studies were conducted in 25-l tanks. On the day of challenge experiment, water in each tank was drained completely and fish were stressed for 30 s. Immediately after stressing, 1 l of water containing appropriate concentration of either bacteria or virus

Table 1 Obligonucleotide primers used in this study

Primer no.	Nucleotide sequence	Target gene
TTC757 TTC758	[Fd]: 5′-TCGTACGAGACATCAAGGAG-3′ [Re]: 5′-AGGAAGGAGGGCTGGAAGAG-3′	β-Actin
TTC921 TTC922	[Fd]: 5′-CACCAAAATCAACGGGACTT-3′ [Re]: 5′-TACTCAGACAATGCGATGC-3′	Cecropin P1 transgene
TTC955 TTC956	[Fd]: 5′-GCGTGGATTGCGGTTTGACT-3′ [Re]: 5′-ATAAGAGAGCAGGGCGAGGA-3′	CF-17 transgene
TTC1251 TTC1249	[Fd]: 5′-GCCTGCTCCTGCTCCTCGCC-3′ [Re]: 5′-CTGGATGGCGATGGCGATGC-3′	mRNA of cecropin P1
TTC1310 TTC1311	[Fd]: 5′-TCTCTACCAGCCTGCTCCTG-3′ [Re]: 5′-CTAGAGGGCTTGTCCCACC-3′	mRNA of CF-17 transgene
TTC1367 TTC1368	[Fd]: 5′-TTCATATGCCAGGCTCAACA-3′ [Re]: 5′-GCTAATGGACGACGCTTTTC-3′	Y chromosome specific sequence

was added to each tank, and the fish were kept for an additional 6 h with constant aeration in the tank. At the end of challenge with pathogens, aeration was stopped and air stones removed. Large volume of constant running water is flooded into each tank and fish were kept for another 4 to 6 weeks till the end of experiment. At the end of the experiment, the total numbers of survival fish were recorded and survival rates calculated to determine the mortality of each family.

Production of All-Male Transgenic Fish

All-male transgenic trout was produced following the method described by Parsons and Thorgarrd (1985). Briefly, eggs were collected from non-transgenic females, exposed to Co^{60} gamma radiation at a dose of 3×10^4 R. The irradiated eggs were fertilized with sperm of F_3 heterozygous transgenic families. The diploidy of the fertilized eggs was restored by suppressing the first cleavage division using hydrostatic pressure and the hatched fry were reared to adulthood. The YY transgenic males were identified by PCR amplification of cecropin P1 transgene and the male was identified by PCR amplification of the Y chromosome specific sequence (Brunelli et al. 2008) using oligonucleotide primers listed in Table 1.

Results

Production, Identification and Characterization of Cecropin P1 and CF-17 Transgenic Rainbow Trout

Linearized cecropin P1 and CF-17 transgenes were transferred into rainbow trout via the sperm-mediated gene transfer method as described in Materials and Methods. The presence of transgenes in P_1 presumptive transgenic animals were determined by PCR amplification of the genomic DNA isolated from fin clips (Fig. 2). About 30 % of the presumptive P_1 transgenic animals were determined to carry cecropin P1 transgene and 25 % of the presumptive transgenic animals carried CF-17 transgene, respectively. At reproductive maturation, each P_1 transgenic fish was mated with counterpart non-transgenic fish to establish F_1 founder families. A total of eight founder heterozygous transgenic families carrying cecropin P1 transgene and nine families carrying F-17 transgene were established. Each family of the founder fish was mated to non-transgenic fish to establish F_2 and F_3 generations.

To detect the expression of cecropin P1 or CF-17 transgene in F_1, F_2 and F_3 generations of heterozygous transgenic progeny, total RNA samples isolated from tissues of liver, muscle and spleen were subjected RT-PCR analysis. Figure 3 shows the representative results of cecropin P1 transgene expression in three randomly chosen F_1 transgenic families. Transgene mRNAs were detected in tissues of liver, muscle and spleen of all eight families of cecropin P1 and nine families of CF-17 fish. To determine whether the expression of cecropin P1 transgene in transgenic fish will affect their growth performance when compared to the non-transgenic fish, the body lengths of several families of F_1 heterozygous transgenic progeny were compared with their non-transgenic counterparts. The representative result of one family is shown in Fig. 4. In this comparison, the average size of non-transgenic fish ranged about 16.0 g and that of transgenic fish ranged about 15.4 g. These results might suggest the expression of cecropin P1 transgene in the transgenic fish family might not affect their growth performance when compared to non-transgenic fish.

Challenge Studies with *A. salmonicida* and IHNV

The antimicrobial activities of F_2 and F_3 heterozygous transgneic progeny were determined by challenging with a bacterial pathogen, *A. salmonicida*, at a dose of 5×10^5 cfu/ml and a viral pathogen, IHNV, at a dose of 5×10^5 pfu/ml. In the challenge studies with *A. salmonicida*, the cumulative

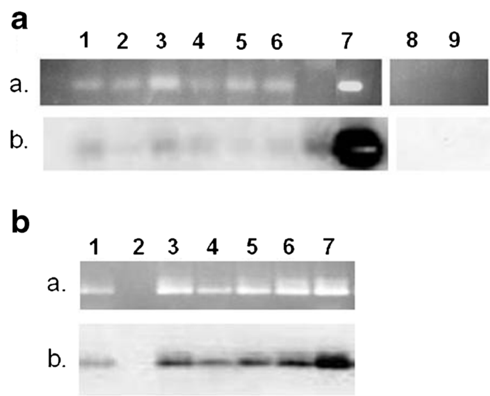

Fig. 2 Identification of transgenic rainbow trout by PCR amplification of genomic DNA from F_1 heterozygous transgenic individuals. **a** Detection of cecropin P1 transgene sequence. *a* PCR products; *b* hybridization signals of PCR products. *Lanes 1–6*, DNA samples from presumptive transgenic fish; *lane 7*, DNA of cecropin P1 transgene construct; *lanes 8–9*, DNA from non-transgenic control fish. **b** Detection of CF-17 transgene sequence. *a* PCR products; *b* hybridization signals of PCR products. *Lanes 1 and 3–6*, DNA samples from presumptive transgenic fish; *lane 2*, DNA sample from non-transgenic control fish; *lane 7*, DNA of CF-17 transgene construct

mortality of control fish ranged 80–85 %, but the cumulative mortalities of different families of F_2 and F_2 cecropin P1 transgenic progeny ranged 12–40 %, except for the family of U6#768 where the mortality was similar to that of the control fish (Table 2). When non-transgenic control fish were challenged with IHNV at the dose of 5×10^5 pfu/ml, the cumulative mortality ranged 82–83 %, but the cumulative mortalities of F_2 and F_3 cecropin P1 transgenic families ranged 4–25 %, except for the family of S8#505 where the mortality was indistinguishable from that of the non-transgenic fish (Table 3). Results of challenge studies of different F_3 heterozygous CF-17 transgenic families with *A. salmonicida* and IHNV are presented in Table 4. By comparing the cumulative mortalities of non-transgenic control fish with different heterozygous families of CF-17 transgenic fish, all nine families of heterozygous CF-17 transgenic fish showed significant resistance to infection by IHNV. Similar results were observed in challenge studies with *A. salmonicida* except families 773 and 900 where challenge studies were not conducted.

The typical pathological signs were observed in morbid transgenic and non-transgenic fry challenged with *A. salmonicida* and IHNV. As shown in Fig. 5, morbid fry challenged with *A. salmonicida* displayed typical pathological signs including lesion of skin ulcers. Microscopically, focal *Aeromonas samonicida* microcolonies were present in multiple tissues, including liver, kidney and heart. When swabs taken sterilely from the kidney of morbid fry were plated on tryptone soy agar medium and cultured at 22 °C, the resulting colonies uniformly showed typical features of *Aeromonas samonicida* with convex morphology and brown pigment.

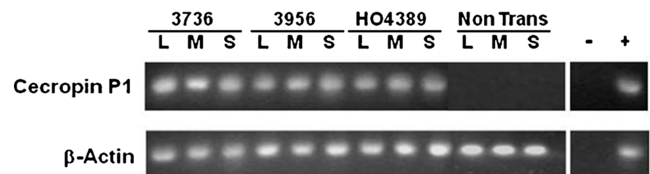

Fig. 3 Expression of cecropin P1 transgene in transgenic rainbow trout. Total RNA samples were prepared from the liver, muscle and spleen tissues of cecrpoin P1 transgenic fish (heterozygous F_1 generation), and were subjected to RT-PCR analysis for detection of cecropin P1 transcript following the method described in Materials and Methods. 3736, 3956 and HO4389 are randomly selected F_1 heterozygous transgenic fish. *L* liver, *M* muscle, *S* spleen; +, plasmid DNA contains cecropin P1 sequence for positive control in PCR; −, PCR reaction in the absence of cecropin P1 DNA

While typical pathological signs including distended abdomen, petechial hemorrhages and exophthalmos were observed in morbid fry challenged with IHNV, microscopic observation revealed extensive necrosis in the internal organs including kidney and liver (Fig. 6).

Development of All-Male Transgenic Rainbow Trout

All-male homozygous cecropin P1 transgenic fish strains were bred from F_3 heterozygous transgenic fish by using the technique of androgenesis developed by Parsons and Thorgarrd (1985). Eight families (S7#375-F073, S9#659-F180, S9#746-F509, U6#768-G410, S8#505-G231, S7#342-F695, A12-944 and A13-831) of all-male homozygous cecropin P1 transgenic fish were successfully bred. Although female transgenic fish were also produced in this operation, these fish were discarded since these fish produced poor quality eggs according to studies by Scheerer et al.

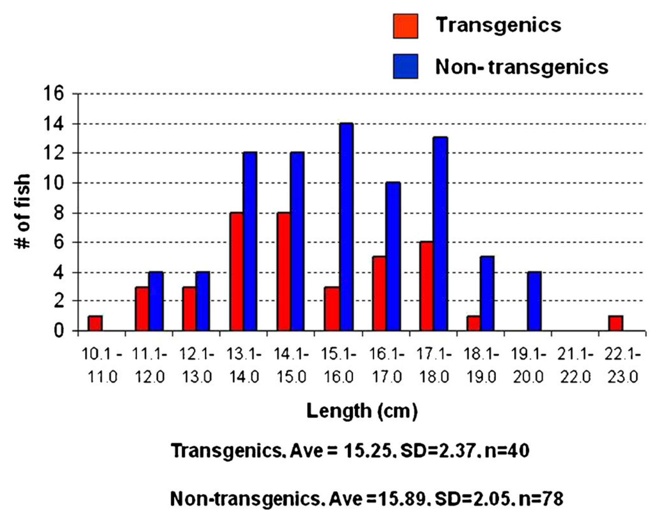

Fig. 4 Body size distribution of transgenic and non-transgenic fish. The body sizes of cecropin P1 heterozygous transgenic fish (F_1 generation) and their non-transgenic sibling controls were measured and grouped into different size classes

Table 2 Mortalities of F_2 and F_3 heterozygous cecropin P1 transgenic fish challenged with *A. salmonicida*

Families	% Mortality (mean±SD)	
	F_2 generation	F_3 generation
S8#Y419	12±3	15±5
S7#375	14±5	18±8
S9#746	20±6	30±5
S7#342	30±3	35±4
S8#505	20±4	26±8
S9#638	10±2	14±8
S9#659	25±6	40±10
U6#768	80±3	79±12
Non-transgenic	80±6	85±8

For each family, challenge was conducted with 30 fish/family (1–2 g body weight)in triplicates and the dose of *A. salmonicida* (5×10^5 cfu/ml) used in each challenge study brings about 80 % mortality in non-transgenic fish

(1991). The expression of cecropin P1 transgene in heart, liver, muscle and spleen tissues of six selected homozygous families was confirmed by RT-PCR analysis. As shown in Fig. 7, various levels of cecropin P1 mRNA were detected in these tissues. Two selected families (namely S7#375-F073 and S7#659-F180) of cecropin P1 homozygous all-male fish were outcrossed to non-transgenic females and their heterozygous progeny were subjected to challenge studies with *A. salmonicida* at the concentrations of 1×10^5 and 5×10^5 cfu/ml, and the results of the challenge studies were presented in Fig. 8. Persistent resistant characteristic to *A. salmonicida* infection was observed in the heterozygous fish derived from both homozygous families out cross to non-transgenic fish.

Table 3 Mortalities of F_2 and F_3 heterozygous cecropin P1 transgenic fish challenged with *IHNV*

Families	% Mortality (mean±SD)	
	F_2 generation	F_3 generation
S8#Y419	25±3	20±6
S7#375	20±5	18±6
S9#746	4±2	10±5
S7#342	15±3	17±4
S8#505	79±8	82±4
S9#638	12±2	11±8
S9#659	20±6	25±9
U6#768	18±5	25±5
Non-transgenic	82±6	83±8

For each family, challenge was conducted in 30 fish/family (1–2 g body weight)in triplicates and the dose of IHNV (5×10^5 pfu/ml)used in each challenge study brings about 80 % mortality in non-transgenic fish

Table 4 Mortalities of F_3 heterozygous CF-17 transgenic rainbow trout challenged with *A. salmonicida* or IHNV

Families	% Mortality (mean±SD	
	A. salmonicida	IHNV
711	15±5	12±2
756	40±6	20±4
773	–	20±2
829	30±6	20±3
850	40±4	40±5
887	55±7	20±4
900	–	15±2
908	20±2	25±2
921	20±3	20±1
Non-transgenic	85±4	82±2

For each family, challenge was conducted with 30 fish/family (1–2 g body weight)in triplicates and the dose of *A. samonicida* (5×10^5 cfu/ml)or IHNV(5×10^5 pfu/ml)in each challenge study brings about 80 % mortality in non-transgenic fish

– not tested

Discussion

In aquaculture industry worldwide, bacterial and viral diseases result in tremendous economic losses annually. Current strategies to control these diseases consist of prophylaxis such as vaccination with inactivated pathogens or recombinant vaccines, medication with chemicals or antibiotics, and eradication of infected populations. Although effective vaccines have

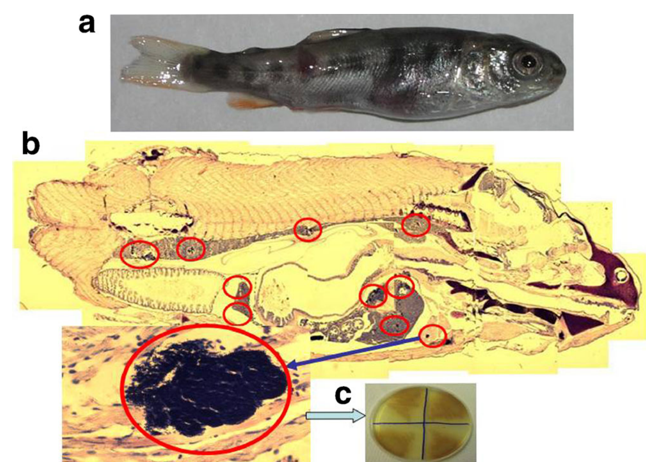

Fig. 5 Fry of non-transgenic rainbow trout infected with *A. salmonicida* exhibited typical pathological signs. Morbid fry showed typical lesion of skin ulcer (**a**). Microscopically, focal *A. salmonicida* microcolonies (indicated by *red circles*) were present in multiple tissues, including liver and kidney (**b**). Occasionally, the bacteria microcolonies were observed in the heart (*inset* in **b**). Swabs taken sterilely from the kidney of morbid fry were plated on tryptone soy agar (*TSA*) and incubated at 22°C. The colonies uniformly showed typical features of *A. salmonicida* with convex morphology and brown pigment (**c**)

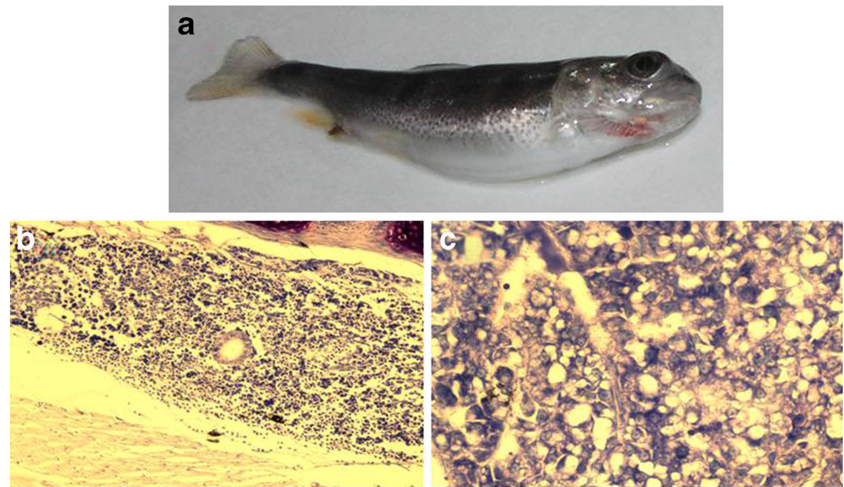

Fig. 6 Fry of non-transgenic rainbow trout infected with IHNV exhibited typical pathological signs. **a** Morbid fry showed clinical signs typical to the IHNV disease, including distended abdomen, petechial hemorrhages, and exophthalmos. Microscopically, extensive necrosis was observed in the internal organs, including kidney (**b**) and liver (**c**)

been developed for selective important fish pathogens over the past decades, the economic cost of developing vaccines and the labor intensiveness of vaccination practice frequently out-weigh the economic benefits. Furthermore, the potential risk of residual levels of antibiotics left in the flesh of fish and the selection of antibiotic-resistant bacterial strains in the aquatic environment argues against the benefit of using antibiotics to control fish diseases. If a genetic trait that will confer fish to be resistant to bacterial, viral or parasitic infection can be identified, manipulation of this genetic trait by transgenesis may present a unique opportunity to resolve the problem of disease outbreak in aquaculture. What genetic trait may confer fish resistant to infection by pathogens?

It has long been recognized that both innate and adaptive immunity systems are required for fish and higher animals to overcome infection by microbial pathogens. While adaptive immunity can protect the host from infection by specific microbial pathogens, it usually requires a longer period of time for the host to develop antigen-specific antibodies and immunologic memory against the specific pathogen. Since fish are living under a lower temperature environment compared to mammals, it will require a longer period of time to develop adaptive immunity to control the initial phase of infection by microbial pathogens (Boman 1995; Bonizzi and Karin 2004). The innate immunity system could serve as the first line of defense to eliminate primary infection by microbial pathogens because the host produces antimicrobial peptides or other small molecular weight compounds with antimicrobial activities within hours upon exposure to pathogens (Boman 1995). Since the identification of the first insect antimicrobial peptides, cecropins, a large body of similar antimicrobial peptides has been identified in a wide variety of organisms throughout the animal kingdom (Hultmark et al. 1980; Boman and Hultmark 1987; Destoumieux et al. 1997; Chiou et al. 2005; Pillai et al. 2005; Syvitski et al. 2005). In vitro and in vivo studies showed that these peptides possess activities against a broad spectrum of fish bacterial viral pathogens (Boman 1995; Hancock and Lehrer 1998; Jia et al. 2000; Chiou et al. 2002; Sarmasik et al. 2002). Studies have also been reported that transfer of functional cecropin B gene into plants resulted in the production of transgenic plants with elevated resistance to bacterial pathogens (Jaynes et al. 1987; Hassan et al. 1993; Jia et al. 1993; Huang et al. 1997; Osusky et al. 2000). These results suggest that transgenic manipulation of antimicrobial peptide genes may lead to the production of fish strains with elevated resistance to bacterial and viral pathogens.

To test this hypothesis, Sarmasik et al. (2002) introduced transgenes of pre-pro-cecropin B, pro-cecropin B, mature cecropin B and cecropin P1 driven by a CMV promoter into Japanese medaka (*Orizias latipas*). The resulting F_2 transgenic progeny displayed significant resistance to *P. fluorescens* and *V. anguillarum* in repeated challenge studies. Like transgenic plants expressing antimicrobial peptide genes (Jaynes

Fig. 7 Detection of cecropin P1 transgene expression in homozygous transgenic fish by RT-PCR analysis. **a** RT-PCR products of cecropin P1 transgene; **b** RT-PCR product of β-actin gene. Total RNA samples were prepared from tissues of heart, spleen, muscle and liver and subjected to RT-PCR analysis for the expression of cecropin P1 transgene following methods described in Materials and Methods. *H* heart, *S* spleen, *M* muscle, *L* liver

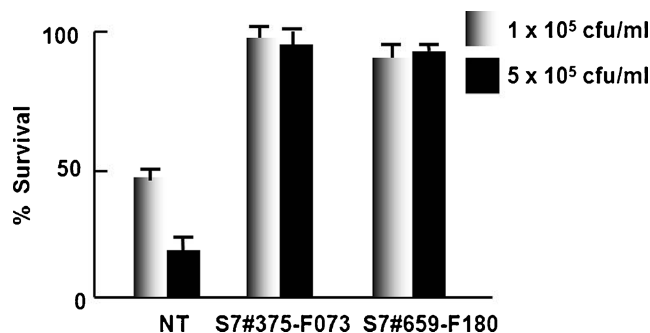

Fig. 8 Percent survival of heterozygous cecropin P1 transgenic fish derived from all-male homozygous fish challenged with *Aeromonas salmonicida*. Triplicate families of heterozygous transgenic fish progeny (50 fish/family of 0.4–0.5 g body weight) and non-transgenic controls were challenged with *A. salmonicida* at doses of 1×10^5 and 5×10^5 cfu/ml as described in Materials and Methods. Morbid fish were collected daily over a period of 2 weeks post-challenge with the pathogen

et al. 1987; Hassan et al. 1993; Jia et al. 1993; Huang et al. 1997; Osusky et al. 2000), transgenic medaka expressing cecropin B or cecropin P1 transgene displayed resistant characteristic to common fish bacterial pathogens.

Will an aquaculture important fish species exhibit the similar resistant characteristic to common fish bacterial pathogens if a cecropin B or cecropin P1 transgene is introduced into the fish by transgenesis? To answer this question, we produced transgenic rainbow trout carrying cecropin P1 or CF-17 transgene fused to the CMV promoter by sperm mediated gene transfer method. PCR analysis of the presumptive P_1 transgenic fish showed that about 30 % of the presumptive P_1 transgenic animals carried the cecropin P1 transgene and 25 % carried the CF-17 transgene. These results are consistent with those reported by other investigators (Powers et al. 1992; Symonds et al. 1994; Sin et al. 2000; Lu et al. 2002). A total of eight F_1 founder heterozygous transgenic families carrying cecropin P1 transgene and nine F_1 heterozygous families carrying CF-17 transgene were established, respectively. Results of RT-PCR analysis confirmed the expression of cecropin P1 and CF-17 transgenes in F_1, F_2 and F_3 heterozygous transgenic progeny.

Fish digestive tract, like the digestive tract of human, is inhabited by large numbers of pathogenic and non-pathogenic microorganisms (Cummings and Macfarlans 1997; Guarner and Malagelada 2005; Ray et al. 2012; Sanchez et al. 2012). Many of these non-pathogenic microorganisms contribute greatly to the digestion of foods, supplying vitamin B complex and other essential nutrients, and enhancing absorption of nutrients by the fish gut (Ray et al. 2012; Sanchez et al. 2012). Since antimicrobial peptides possess bactericidal activity to a large spectrum of microorganisms, it is conceivable that cecropin P1 and CF-17 transgene expressed in the transgenic fish may kill the beneficial microorganisms in the gut and thus affect the growth performance of the fish. When the patterns of body size distribution of several F_1 heterozygous transgenic families expressing cecropin P1 transgene were compared to that of non-transgenic siblings, the patterns of body size distribution of transgenic fish were not significantly different from to those of the non-transgenic fish. These results suggest that the expression of cecropin P1 transgene in the transgenic fish may not affect the population of the beneficial microorganisms inhabiting in the fish digestive system. Similar observation has also been reported by Dunham et al. (2002) in F_1 heterozygous transgenic channel catfish (*Ictalurus punctatus*) expressing cecropin transgenes.

Types of pathogens, routes of pathogen entry, and the virulence of pathogens are three critical factors in pathogen challenge studies (Michel 1980; Adams et al. 1987). Since *A. salmonicida* and IHNV are two well-recognized aggressive pathogens infecting salmonid fish species, we chose these two organisms as target pathogens for the challenge studies to determine the antimicrobial activity of cecropin P1 and CF-17 transgenic fish. In the challenge studies, *A. salmonicida* at the concentration of 5×10^5 cfu/ml or IHNV at the concentration of 5×10^5 pfu/ml were introduced into fish of 1–2 g body weight by immersion because this method could provide the most natural route of pathogen entry into the fish (McCarthy 1983; Hjeltnes et al. 1989). In repeated challenge studies, while exposure of non-transgenic fish to 5×10^5 cfu/ml of *A. salmonicida* or 5×10^5 cfu/ml of IHNV resulted in about 80 % of cumulative mortality, exposure of F_2 or F_3 transgenic families of cecropin P1 transgenic fish to *A. salmonicida* brought about 10–40 % cumulative mortalities and 4–20 % cumulative mortalities to IHNV. Similar degrees of protection from infection by both pathogens were also observed in CF-17 transgenic families. It is of interesting to note that while most families of F_2 and F_3 cecropin P1 transgenic fish displayed resistant characteristic to both pathogens, family U6#768 failed to show resistant characteristic to *A. salmonicida* and family S8#505 failed to display resistant characteristic to IHNV even though both families expressed cecropin P1 transgene. The reason for this dichotomy requires further investigation. Nevertheless, the overall results from the current study are in good agreement with the in vitro studies reported by Chiou et al. (2002) where they reported that synthetic cecropin B and CF-17 were effective in killing *A. salmonicida* and various fish RNA viruses including IHNV.

In summary, we demonstrated in this study the production of strains of stable transgenic rainbow trout expressing cecropin P1 or CF-17 transgene by sperm-mediated gene transfer method. These transgenic fish displayed elevated resistant characteristic to infection by *A. salmonicida* and IHNV. By employing the technique of androgenesis, homozygous all-male (YY) transgenic families have been bred in one generation; and these transgenic stocks can be preserved via sperm cryopreservation. Dunham et al. (2002) reported previously the production of transgenic channel catfish (*Ictalurus punctatus*) harboring cecropin B transgenes

exhibiting elevated resistant to bacterial pathogen in the F_1 generation. Therefore, results presented in this paper, together with those of Dunham et al. (2002), clearly point to the possibility of producing disease-resistant fish strains for aquaculture by transgenic manipulation of antimicrobial peptide genes.

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