

Growth Response to a GH-Autotransgenesis in Common Carp *Cyprinus carpio*

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Abstract

Autotransgenic manipulation with a growth hormone (GH)-construct is a potential approach to improving the growth rates of farmed fish. Here, we present the generation of GH-autotransgenic common carp *Cyprinus carpio* carrying a transgene comprised of the carp homologous GH gene and a β -actin regulator. Autotransgenic carp showed similar viability to their non-transgenic siblings. Early growth characteristics of founder autotransgenic carp up to 50 days postfertilization were highly variable among individuals; *i.e.*, some fish exhibited significant growth depression, while others showed dramatic acceleration of growth, achieving greater than sixfold increases in body weight relative to their non-transgenic counterparts. Stimulated growth performance became more notable with age and many transgenic individuals of the largest class reached 5 kg within 8 or 9 months, which is at least 10 times heavier than the average body weight of communally grown non-transgenics. Four of six founder transgenic males were successful in passing the transgene to their F₁ offspring with frequencies ranging from 19 to 36%. Growth stimulations were also persistent in all F₁ progeny groups examined.

Key words: *Cyprinus carpio*, Common carp, Autotransgenic, Growth hormone-transgenesis, Growth enhancement

Introduction

Manipulation of growth traits through growth hormone (GH)-transgenesis has received a lot of attention as a potential means of overcoming the drawbacks of traditional selective breeding of farmed fishes despite the numerous ecological risks associated with GH-transgenic geno- and phenotypes that remain to be addressed (Devlin et al., 2006; Nam et al., 2007). Early research in fish GH-transgenesis has largely focused on the use of genetic elements of non-piscine origins (*i.e.*, viral or mammalian). Moreover, even if piscine genetic materials were used, the regulators and/or structural GH genes tended to originate from species distantly related to the recipient host (Gong et al., 2007). As a result, the growth responses of the transgenic strains tended to be modest or weak (Gong et al., 2007; Nam et al., 2008).

Over the past decade, many researchers have suggested that

homologous gene constructs are more effective than distantly heterologous ones for GH-transgenesis in fish (Zbikowska, 2003). The effectiveness of GH-transgenesis using completely homologous genetic elements for both the promoter and structural gene (*i.e.*, GH-autotransgenesis) was clearly demonstrated for the first time in the mud loach *Misgurnus mizolepis* (Nam et al., 2001). This achievement has now been repeated via many ongoing autotransgenic manipulations in a variety of fish species including tilapia *Oreochromis niloticus* (Maclean, 2003), blunt-snout bream *Megalobrama amblycephala* (Fu et al., 2005), and Indian major carp *Labeo rohita* (Rajesh and Majumdar, 2005). However despite these efforts, no clear demonstration of GH-autotransgenesis has yet been reported.

Common carp *Cyprinus carpio*, a worldwide cosmopolitan species, is one of the most important aquaculture species in

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the world, with a global industry value of more than 3 billion USD. Not surprisingly, this species has a target for a variety of transgenic manipulations, particularly GH-transgenesis. The earliest transgenic trial for the growth enhancement of carp was carried out using a transgene comprising a viral promoter (RSV-LTR) fused to salmon GH cDNA (Chen et al., 1993). However, most of these transgenic lines showed less than 50% growth increases (Chen et al., 1993; Devlin et al., 1994). A much better growth performance was achieved for GH-transgenic carp (at least twofold relative to non-transgenics) using an “all-cyprinid” transgene construct, for which the regulatory component and GH structure gene originated from a closely related grass carp *Ctenopharyngodon idella* and the common carp, respectively (Wu et al., 2003). Another attempt at GH-transgenesis used the carp β -actin promoter spliced to carp GH cDNA fused to a Chinook salmon *Oncorhynchus tshawytscha* polyadenylation signal, which resulted in improved growth of transgenic carp during the winter seasons (Hinits and Moav, 1999). Overall, these reports suggest that GH-transgenesis with homologous constructs produce more desirable growth responses. However, despite its importance, to date no complete autotransgenic carp line exists. We recently reported the development of autotransgenic common carp in our review article (Nam et al., 2008). Here, we provide a detailed report of the growth characteristics of the transgenic common carp founders and their F_1 progeny.

Materials and Methods

Generation of autotransgenic common carp and early viability assessment

To construct the GH-transgene, a 2.5-kb portion of the common carp β -actin regulatory region, including the non-translated exon I and intron I, was spliced upstream of the 2.1-kb carp growth hormone genomic gene (*pca β -actGH*). Linearized *pca β -actGH* resuspended in 0.1 mM Tris-Cl at a concentration of 100 μ g/mL was microinjected into one-celled embryos obtained from artificial fertilization. Injected embryos were maintained in an incubator at 25°C until hatching. Approximately 1,800 embryos were injected and a similar number of non-injected embryos were prepared for the control sibling group. Hatching success and early survival rate were estimated with 55 randomly chosen embryos in triplicate. After hatching, larvae from the injected and non-injected groups were reared in 50-L recirculating tanks. Fish were fed artificial carp feed (40% crude protein). At 2 weeks post-hatching, fish were transferred to two separate 120-L tanks and further grown to 50 days postfertilization for PCR screening of the transgene. During this period, early viability was estimated weekly for both microinjected and non-injected groups.

PCR typing of the transgene

At 50 days postfertilization, caudal fin tissue (~50 mg) was obtained from each putative transgenic individual that was heavier than the average individual from the non-injected control group. Genomic DNA was prepared using a conventional sodium dodecyl sulfate/proteinase K method followed by organic extraction and ethanol precipitation (Nam et al., 2001). A 1- μ g aliquot of purified DNA served as a template for PCR amplification of the transgene using a pair of primers specific to either the β -actin regulator (*ca β -actF*: 5'-ACATGGTCA-CATGCTCACTG-3') or the GH gene (*caGHR*: 5'-ACACCTGCACCAGCTGGCTG-3'). The thermal cycling conditions were as follows: 33 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with an initial denaturation step at 94°C for 3 min and a final elongation step at 72°C for 5 min. Fish that were PCR-positive for the construct were selected for further examination of their growth performance until sexual maturity. For the non-transgenic group, representative individuals whose body weights were closest to the average body weight of the control group were selected.

Growth trial of founder generation transgenic fish

Communal rearing was carried out to examine the differential growth rates between GH-transgenic and non-transgenic carp under the same culture conditions. Selected transgenics ($n = 48$) and non-transgenics ($n = 24$) were marked with fluorescence tags (Northwest Marine Technology Inc., Shaw Island, WA, USA) as described previously by Nam et al. (2001), allocated together into a large rectangular tank (3 M \times 10 M \times 1 M = W \times L \times H) and grown to 8 months of age. During the growth trial, water quality was controlled using a semi-recirculation system with a 30% daily water exchange. The water temperature was 25 \pm 2°C throughout the experiment. Fish were fed artificial carp feed 6-8 times per day on an *ad libitum* basis. Body weight was measured every month and mortality was checked at the same time.

Germ-line transmission of the transgene and early growth of the F_1 generation

Nine-month-old transgenic males ($n = 8$) were given an intraperitoneal injection of carp pituitary extract (Sigma, St. Louis, MO, USA) at a dose level of 2 mg/kg body weight. After 24 h, milt was hand-stripped from six of the eight males injected. Artificial insemination was carried out using the wet method and the resultant fertilized eggs were kept at 25°C until hatching. A non-transgenic progeny group was also generated using the same methods to serve as a control. Hatched larvae (~1,200 larvae) from each cross were transferred to a rectangular tank (1.2 M \times 3 M \times 0.5 M = W \times L \times H). At 1 week post-hatching, a random sample of 36-48 fry was collected from each cross and subjected to PCR screening for the trans-

gene using the same conditions as described above. Genomic DNA was prepared from whole body fry. After 2 weeks, many individuals were showing large body sizes that clearly deviated from the normal distribution of body weights seen within the non-transgenic group. Of the fast-growing fishes, 36-40 randomly chosen individuals per group were verified for their transgenic status by PCR and further subjected to early growth trials along with a representative non-transgenic sibling group for 2 months.

Results and Discussion

Percent hatching success (percentage of eggs injected) and early survival up to yolk sac absorption (percentage of hatched larvae) of the microinjected groups as determined from 55 randomly chosen embryos were $36 \pm 3\%$ and $69 \pm 4\%$, respectively. Both of these values were significantly lower than those of the non-injected control embryos ($80 \pm 5\%$ for hatching and $78 \pm 4\%$; $P < 0.05$). Therefore, of the 1,800 microinjected embryos, 657 hatched and 453 of these larvae survived until yolk sac absorption was complete. This decreased viability of microinjected embryos has been reported by numerous studies, and the present scores were generally in agreement with those previously reported (Nam et al., 2007). In total, 441 individuals were viable at 1 month post-hatching and 435 individuals survived to 50 days of age, which was not significantly different from the viability of non-injected groups during the same period ($P > 0.05$).

At 50 days postfertilization, the presence of the transgene in presumed transgenic founders differed substantially according to body-size classes when assessed by PCR screening. In the small-sized group (body weight range, 0.1 to 10.0 g), the frequency of individuals harboring the *pca β -actGH* construct was 9.8% (five PCR-positive individuals of the 51 fish tested). Notably, only 3.5% of the medium-sized group (range, 10.1 to 25.0 g) showed the transgene (10 of 287 individuals). Conversely, the large-sized group (heavier than 25.1 g) exhibited a significantly higher transgene incidence (55.7%; 54 of 97 individuals) than the other two groups. Moreover, several PCR-positive individuals identified from the large-sized group exhibited extraordinarily heavy body weights that clearly deviated from the normal distribution of body weights in the non-injected group. Specifically, some transgenic fish belonging to the largest size class weighed more than 140 g, which is at least 6-7 times heavier than the average body weight of the non-injected group (20.8 ± 3.5 g). These data suggest that the early growth of this species was highly responsive to transgenesis with the *pca β -actGH* construct. Note, however, that several presumed founder fish showing quite low weights were clearly PCR-positive for the construct, while approximately 7% of the large-sized fish were PCR-negative. Detection of the transgene in such slow-growing fingerlings could be explained by an inhibitory effect resulting from the

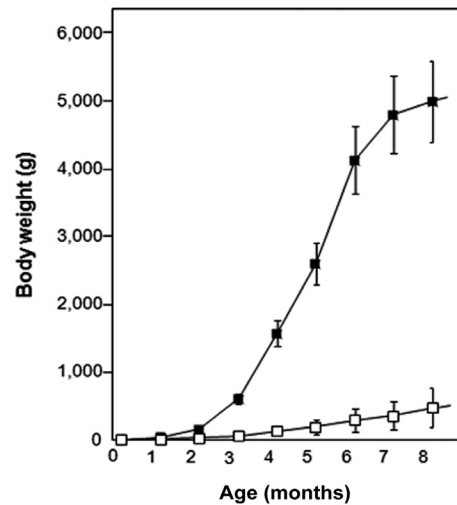


Fig. 1. Body weight growths (mean \pm SD) of founder autotransgenic carps (closed squares) along with non-transgenic siblings (open squares) as function of age up to 8 months under the communal culture conditions.

overexpression of GH during early development (Devlin et al., 1995; Nam et al., 2002). The relatively high frequency of small-sized PCR-positive fingerlings showing abnormal morphologies may support this hypothesis (photograph not shown). Another plausible but unproven assumption is that the transgene integrated into a specific site within the host genome that resulted in an undesirable position effect (Maclean et al., 1987; Hackett and Alvarez, 2000). Detailed evaluations of the transgenic status of these fish, particularly focusing on transgene copies and integration sites, are required to test these hypotheses. Conversely, the occurrence of PCR-negative fish in the large-sized group could be explained by either a mosaic distribution of the transgene across tissues or an extremely high level of mosaicism in the fin tissues that were chosen for PCR screening. Genetic mosaics are often reported to result from microinjected embryos (Nam et al., 2007).

The growth trial during which fish were communally reared in the same tank revealed very interesting results (Fig. 1). Transgenic founders still exhibited extensive variation in body weights. On average, transgenics surpassed their non-transgenic siblings at as early as 2 months of age. The differences between these groups increased greatly with age. By 4 months, the transgenic group exhibited an average body weight of 1.6 kg, which was 12-fold that of the communally grown non-transgenic group (126 g). This difference was even more pronounced at 6 months, at which point the transgenic group showed an average body weight of 4.1 kg, while that of the non-transgenic group was 286 g. Many transgenic carp individuals exceeded 5 kg in body mass at 8 months of age, although several transgenic founders showed no further growth acceleration during this phase, possibly due to suboptimum culture conditions. Both the transgenic and non-transgenic

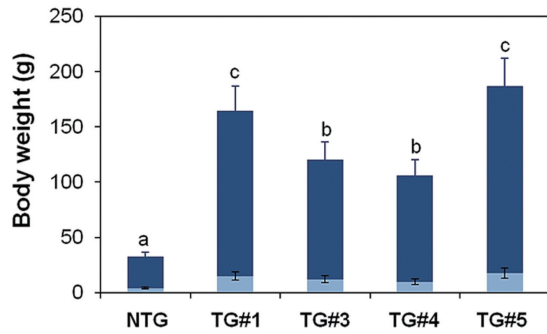


Fig. 2. Weight gains during ages from 2 weeks (light blue) to 2 months (dark blue) in F1 autotransgenic carp strains and non-transgenic siblings (averaged from four non-transgenic crosses). Standard deviations were noted by T bars and the same letters on histograms indicate no significant difference as assessed by ANOVA at $P = 0.05$. NTG, non-transgenic; TG, transgenic.

groups showed greater than 80% survival during the communal tank growth trial. These data suggested that the growth traits of this species could be engineered through an autotransgenic manipulation without any significant adverse effects on viability. Furthermore, the present GH-autotransgenesis was much more effective toward the growth response in carp than previous attempts at transgenesis using heterologous transgene constructs (Fu et al., 2005). Nevertheless, this pilot examination should be followed by further efforts to address many remaining issues associated with growth performances and other production characteristics. Such experiments should include examinations of growth performance under more realistic culture conditions (*i.e.*, commercial or semicommercial scales in ponds). Growth trials in separate tanks comparing transgenics and non-transgenics under intensive culture conditions may also serve to normalize or validate the fold differences observed herein. In addition, long-term monitoring would be valuable to examine whether autotransgenic carp may present any gigantisms beyond normal body size, as was

observed in an autotransgenic mud loach (Nam et al., 2001).

Although the present study was limited to a few transgenic founder males, no notable alteration of their reproductive capacity was found in terms of milt production (data not shown). However, significant depression or retarded gonad development has been reported previously in fast-growing transgenic carp harboring an 'all-cyprinid' GH construct (Fu et al., 2005). Thus, further evaluations of reproductive performance of large numbers of autotransgenic carp should be conducted for both sexes. Artificial insemination between milt from transgenic males and eggs from normal females resulted in fairly good scores for both fertilization rate and hatching success, which were not different from those in control crosses using normal gametes (Table 1). Transgene inheritance to the subsequent generation was detected in four of six crosses as judged by PCR typing of F₁ larvae. However, as expected, all of the founder transgenic males were determined to be mosaics, as evidenced by a germ-line transmission frequency lower than 50% (Table 1). All four F₁ progeny groups showed a stimulated pattern of body weight increase during the early stages. Although strain-specific differences were observed during early growth, many transgenic individuals belonging to the F₁ groups could be distinguished from their non-transgenic siblings by the naked eye at 2 weeks of age. From the growth trial up to 2 months of age, weight gains in the transgenic groups ranged from 3.6- to 6.3-fold those of the non-transgenic groups, suggesting that the growth response to the present GH-transgenesis is reproducible in subsequent generations (Fig. 2). The results of this study advocate the use of the autotransgenic strategy for GH-transgenesis of other farmed fish species. Further studies are needed to examine the stable inheritance of geno- and phenotypes through subsequent generations. In addition, several breeding strategies, including chromosome-set manipulations followed by field tests, are needed to select the most desired strain of the autotransgenic carp (Nam et al., 2004; Kapuscinski, 2005).

Table 1. Germ-line transmission of growth hormone transgene from founder autotransgenic carp males to F1 progeny

Group	Fertilization rate (%)	Hatching success (%)	Early survival up to yolk sac absorption (%)	Incidence of transgene (%)
Non-transgenic cross	96.5 ± 2.7	80.3 ± 5.8	82.5 ± 5.5	-
Transgenic male #1	97.1 ± 3.8	78.5 ± 6.5	80.5 ± 6.4	19.1
Transgenic male #2	95.1 ± 4.7	82.1 ± 4.6	78.5 ± 6.5	0.0
Transgenic male #3	93.8 ± 4.4	76.6 ± 5.9	80.1 ± 3.3	36.1
Transgenic male #4	92.8 ± 5.1	81.5 ± 4.9	79.8 ± 6.7	23.7
Transgenic male #5	93.0 ± 4.9	79.7 ± 8.9	82.1 ± 4.5	25.0
Transgenic male #6	93.7 ± 5.5	80.2 ± 3.4	80.8 ± 5.9	0.0

Mean ± SDs for fertilization rate and hatching success were based on triplicate examinations using 110 randomly chosen embryos, while the early survival up to yolk sac absorption was expressed as percentage of hatched larvae. Scores for non-transgenic cross were averaged values from the six crosses. No statistical difference was detected based on ANOVA at $P = 0.05$. Incidence of transgene was estimated by PCR analysis using at least 36 individuals from each cross.

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