Genetic analysis of "all-fish" growth hormone gene trans ferred carp (*Cyprinus carpio* L.) and its F_1 generation

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Abstract **Recombinant "all-fish" growth hormone gene (*GH*) was microinjected into the fertilized eggs of carp. A comparison between the growth traits of transgenics and non-transgenics was carried out, and the transgenic individuals with significant "fast-growing" effect were successfully gained. A comparison on the reproductivities was also given out between the transgenics and their non-transgenic siblings, and showed that the reproductive capacity of transgenics was substantially equivalent to those of the non-transgenics. On the other hand, the genetic separation and the characteristic distribution of the F_1 generation were genetically analyzed, which gave solid evidence for the hypothesis that 2—3 chromosomes are integrated with transgene. In addition, the distinct biological effects for multi-site-integrated transgenes were further discussed. The present study opens a door for the breeding of "fast-growing" transgenic fish.**

Keywords: "all-fish" growth hormone gene, carp, genetic analysis, breeding.

Since the first batch of transgenic fish was born in China[1,2], many laboratories all over the world have turned to the study of transgenic fish, in order to gain new farming strains with the traits of "fast-growing" or "anti-adversity"[3—6]. Compared with the traditional approaches such as interspecific hybridization breeding, transgenic breeding avoids the reproductive isolation between two different species. Since more and more manipulated genes are available for gene transfer, it is hopeful for us to shorten the breeding period through directional genetic breeding. Meanwhile, the transgenic breeding can bring new ideas to bioreactors. Considering the present status of transgenic animals, "all-fish" *GH* transgenic fish was most likely to become the successful example for the breeding of transgenic animals.

It is a complicated process that the integration and expression of transgenes occur in host fish. The study of "model of transgenic fish"[7] suggested that the transgenes were randomly integrated into the host genome. First, it is a gradual process in the aspect of integration timing, that is to say, the integration does not uniformly occur in various tissues and cells, resulting in the transgenic mosaicism in host fish. Second, it is a diverse event in the aspect of integration space, that is to say, the integration does not occur at the specific site of the host genome, resulting in the polymorphism of integration sites. Three categories of transgene integration could be classified by their influence on the host genome, they are functional integration, silent integration and toxic integration. It is of important consequence in the breeding of transgenic fish to understand the genetic law of transgene and to gain the homozygous transgenics with "functional integration".

The genetic analysis and distribution of achieved traits in P_0 generation of "all-fish" *GH* transgenic carp and F_1 generation were studied here.

1 Materials and methods

(ⅰ) Fish for the examination. Yellow River carp (*Cyprinus carpio* L.) were supplied by the Henan Institute of Aquaculture, Henan Province.

(ⅱ) Gene construct pCAgcGHc. "All-fish" gene construct pCAgcGHc was the recombinant construct of grass carp (*Ctenopharyngodon idellus*) growth hormone cDNA (*gcGHc*) driven by β-actin gene promoter of common carp (*Cyprinus carpio* L.), which was modified from the construct pCAgcGH[8]. pCAgcGHc was digested with *Eco*RⅠ and *Hin*dⅢ for gene transfer (fig. 1).

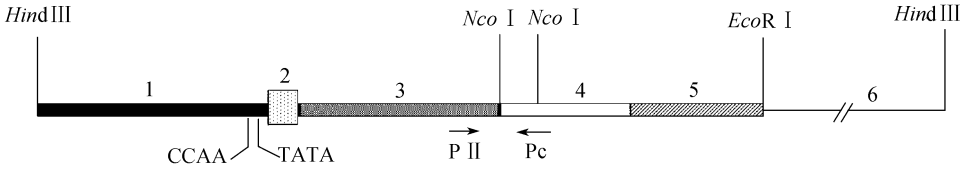

Fig. 1. Structure of pCAgcGHc. 1, 5′ flanking sequence of common carp β-actin gene; 2, first exon of common carp β-actin gene; 3, first intron of common carp β-actin gene; 4, cDNA sequence of grass carp GH gene; 5, 3′ flanking sequence of grass carp GH gene;, 6, pUC118; PⅡ/Pc, primers for PCR amplification.

(ⅲ) Gene transfer. Around the late April, fertilized eggs of Yellow River carp were obtained by artificial spawning and insemination. Microinjection was carried out before the first cell division according to the method of Zhu et al.[7].

(ⅳ) Breeding of transgenic fish. P_0 generation of

transgenics was produced by microinjection with *CAgcGHc* into the fertilized eggs of Yellow River carp. 500 gene-transferred fishes were farmed in a pond of 700 m^2, and so did the non-manipulated fishes. F_1 generation of transgenics was produced as follows, P_0 transgenic ♂ (sperm revealed to be *CAgcGHc* positive) × non-transgenic ♀. 4000 F_1 transgenic fishes were farmed in a pond of 2640 m^2 and the controls were farmed under the same situation. Fish feed with no growth additive was bought from the Dade Feeding Corporation, Henan Institute of Aquaculture.

(ⅴ) Count of brood amount, fertility rate and hatchability. Brood amount, fertility rate and hatchability were figured out as follows: weight of the mature ovary/body weight, number of the embryos at gastrula stage/number of the fertilized eggs and number of the hatched fries/number of the fertilized eggs, respectively. The fertility rate and hatchability of transgenics were figured out through sampling the fertilized transgenic eggs (transgenics × non-transgenics), and those of the controls were figured out through sampling the fertilized eggs of 5 pairs of non-transgenic parents.

(ⅵ) Detection of transgene. Tail fins were cut from the gene-transferred fish and total DNAs were extracted with routine method. PCR was performed to examine the existence of *CAgcGHc*-transgene. Two primers for PCR were PⅠ (5′-TGGCGTGATGAATGTCG-3′) and Pc (5′-AACACGTATGACTGC-3′). The PCR reaction (25 μL in total) contained each of primer 25 pmol, each of dNTP 500 pmol, template DNA 100—500 ng and 0.5 U Taq DNA polymerase. The reaction process included a pre-denaturing at 94℃ for 2 min, 20 cycles of denaturing at 94℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ for 1 min, and an extension at 72℃ for 5 min. The PCR products were separated by 0.8% agarose gel electrophoresis in TAE buffer.

(ⅶ) Analysis on the integration sites of transgene. PCR was carried out with the F_1 generation embryos constructed from the cross between transgenic and non-transgenics. According to the Mendelian manner, the transgene-positive rate (P) of the F_1 generation should be $P = 1–(1/2)^n$, in which n refers to the number of non-homogeneous chromosomes integrated with transgene. If transgene(s) are integrated at the homogeneous chromosomes of transgenic, P equals 1. The positive rate of F_1 generation was examined for goodness-of-fit-test (degree of freedom (d_f) =1).

2 Results

(ⅰ) Distribution of the body weights of P_0 generation. After 120-d feeding, 324 transgenics and 359 non-transgenics were sampled for body weighing. Results suggested that the body weights of non-transgenics were normally distributed, in which the even body weight was 863 g, the heaviest was 1414 g and the lightest was 264 g. And the body weights of transgenics were far from normal distribution, in which the heaviest body weight was 2750 g and the lightest was 84 g. Among the transgenics, 8.7% (29 against 324) got the body weights higher than the heaviest one of the non-transgenics and 6.4% (21 against 324) were higher than 2 kg (fig. 2).

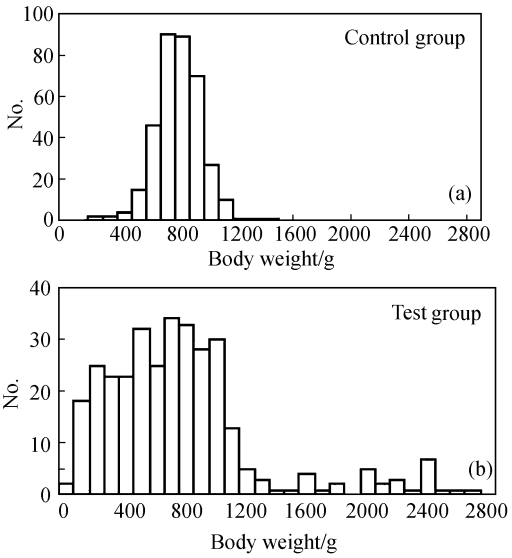

Fig. 2. Distribution of body weights of P_0 transgenics.

(ⅱ) Apparent performance of transgenic fish. The significant "fast-growing" individuals of the P_0 transgenic fish appeared to be very stocky in body shape, and have much thicker muscles on backside and obvious hunch behind the head (fig. 3). A part of the transgenics was shown to be abnormal on the eyes, squamae and backbone. It might be due to the manipulation harm of microinjection or the "toxic integration" of the transgene, which resulted in the absence or errors in the expression of housekeeping genes during embryogenesis and ontogenesis.

Fig. 3. *CAgcGHc* transferred Yellow River carp.

(ⅲ) PCR amplification of transgene in germ cells. The "fast-growing" individuals which were *CagcGHc* positive in tail fins were selected for artificial spawning and insemination. PCR was carried out in each spermary or ovary with the primers PⅠ and Pc. Among 8

female fish, 4 were artificially spawned successfully and all of them were shown to be transgene positive in unfertilized eggs. Among 3 male fish, 2 were shown to be transgene positive in sperm. The transgene positive ones in gonad were used to produce F_1 generation of transgenic fish.

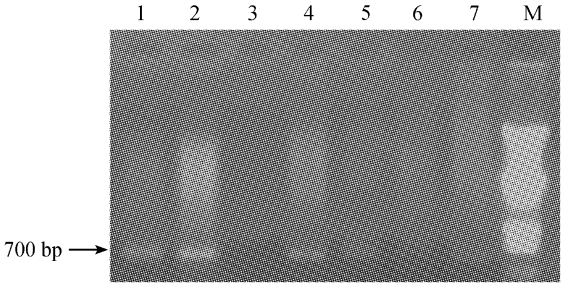

Fig. 4. Detection of transgene in gonads by PCR. 1—3, DNA samples of sperm; 4—7, DNA samples of unfertilized eggs; M, λ DNA/*Hin*dⅢ + *Eco*RⅠ.

(ⅳ) Reproductive parameters of transgenic fish. Brood amount, fertility rate and hatchability of the 3-year-old transgenic fish were counted. Results showed that the absolute eggs amount of transgenics was similar to that of the non-transgenics, while the brood amount of transgenics was significantly lower than that of the non-transgenics (table 1), which resulted from the increasing of body weights of transgenics. Meanwhile, the fertility rate (>80%) and hatchability (>60%) of transgenics were slightly lower than those of the non-transgenics but the difference did not get statically significant (table 2). It was concluded that the reproductive capacity of transgenics was substantially equivalent to those of the non-transgenics.

Table 1 Brood amount of the transgenics and controls

	Control group			Transgenic group			
	1	2	3	1	2	3	4
Body weight /kg	2.66	1.98	1.21	5.58	4.15	4.53	3.14
Brood weight /kg	0.56	0.35	0.25	0.11	0.60	0.44	0.19
Brood amount (%)	21.05	17.51	20.66	1.97	14.46	9.71	5.94

Table 2 Fertility rate and hatchability of transgenics and controls

	Control group	Transgenic group					
	♀×♂	♀1	♀2	♀3	♀4	♂1	♂2
Fertility rate (%)	95.62	90.90	89.12	84.68	90.94	95.98	97.17
Hatchability (%)	86.08	68.59	65.68	76.28	83.28	68.16	72.17

(ⅴ) Integration sites of transgene. The 4 female transgenics shown to be transgene positive in eggs and 2 male transgenics shown to be transgene positive in sperm were artificially fertilized with non-transgenic controls, respectively. After hatching, 43 fries of each combination were sampled for total DNA extraction and PCR amplification was used to examine the transmission of transgene from one generation to next generation. 31, 38, 34 and 37 fries were detected to be transgene positive in the offspring of the 4 female transgenics, respectively, and 37 and 38 fries were detected to be transgene positive in the offspring of the 2 male transgenics, respectively. It was deduced that 2—3 non-homologous chromosomes in the gonad cells were integrated with transgenes, and following χ^2 verification suggested that the number of integration sites coincided with the number in theory (table 3).

Table 3 *CAgcGHc*-positive rates and No. of integration sites in F_1 transgenics

	♀1	♀2	♀3	♀4	♂1	♂2
Positive rate (%)	72.09	88.37	79.07	86.05	86.05	88.37
No. of integration site	2	3	2	3	3	3
χ^2	0.194	0.029	0.379	0.083	0.083	0.355
$\chi^2 - \chi^2_{0.05}$	$< 0^*$	< 0	< 0	< 0	< 0	< 0

$^* \chi^2_{0.05} = 3.841$ ($d_f = 1$).

(ⅵ) Distribution of the body weights of F_1 transgenics. After 80-d feeding, 300 F_1 transgenics and 300 F_1 non-transgenics were randomly sampled for body weighing. Results showed that both the body weights of transgenics and non-transgenics were normally distributed (fig. 4). The transgenics and non-transgenics were (260.40± 22.47) g and (417.89±79.72) g on an average, respectively, and the coefficients of variability (CV) of them were 8.6% (22.47/260.40) and 19.1% (79.72/417.89), respectively. It was very dramatic that some of the transgenics attained the body weight of 800 g ($\overline{X}$ +4.8S), while the smallest individual was 220 g ($\overline{X}$ –2.5S).

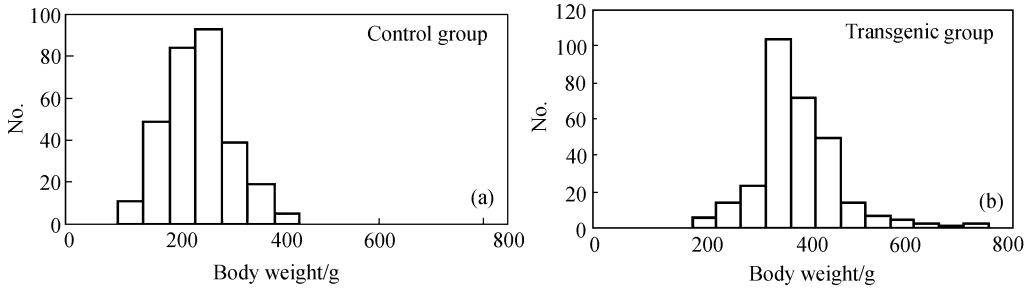

Fig. 5. Distribution of body weights of F_1 transgenics.

3 Discussion

The growth polymorphism of the P_0 transgenic Yellow River carp showed that the integrate sites of transgene in host genome can offer several different biological effects on the phenotype of host fish. The distribution of body weights of the P_0 transgenic fish showed that only 8.7% got significant "fast-growing" trait, while some individuals had even a very slow growth rate or were freakish. In general, there are two preconditions for the "functional integration" of transgene: first, the transgene could be normally expressed; second, the integration and expression of transgene do not interrupt the normal function of the housekeeping genes in host genome. "Functional integration" is a rare event during the process of transgene integrating, while most of the integrations are involved in "silent integration (when integration occurs at the region of heterochromosome)" or "toxic integration (when integration occurs at the region of euchromosome)". Hereby, to gain the individuals with "functional integration" needs large numbers of manipulated samples, which has become one of the limiting factors for the breeding of transgenic mammals. For example, the pathological symptoms of gastric ulcer, pneumonia, etc. or even infertility were shown in *GH*-transgenic pigs, and it is also difficult for the researchers to gain transgenic sheep and goat with goal traits[9]. The technique of site-specific integration of transgene, known as gene targeting, will probably solve this problem.

As to the transgenic fish, it has been mentioned above that the integration sites of transgene are numerous and the inheritance of transgene is complicated, so that the breeding of transgenic fish requires the extensive genetic analysis of the integration sites of transgene. It was reported that both *in situ* hybridization on chromosomes and plasmid rescue were used for analyzing the DNA sequence and flanking sequence of the integration site[10, 11]. Here we reported the genetic analysis of the integration site, which suggested that 2—3 chromosomes of the host Yellow River carp were integrated with transgenes or maybe several separated transgenes were integrated into the linked sites of one chromosome. This result lays a foundation for further analysis of distinct biological effects for different integration sites.

Due to the positional effects of integration sites, the biological effects for different integration sites may be quite different. The comparison of growth performance between F_1 transgenic Yellow River carp and the controls revealed that the transgenics showed significant "fast-growing" trait, and some huge individuals were found in the transgenic group. The body weights of the transgenic group are normally distributed just the same as that of the controls, while the coefficient of variability (CV) of the body weight distributions of the former group is much higher than that of the latter. The results suggest that the expression products of *gcGHc*-transgene in F_1 transgenics are capable of stimulating the growth, but the functional performances are different with different integration sites, which results in the case that the distribution of body weights ($\overline{X}-2.5S$, $\overline{X}+4.8S$) shifts a lot towards the right. It is believed that extra-huge individuals have been produced by highly-efficient integration or several highly-efficient integrations of transgene and the coefficient of variability (CV) of the body weights of transgenic group is affected by the separation of transgene and the positional effects of transgene integration, which results in the dispersed distribution of their body weights.

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References

1. Zhu, Z., Li, G., He, L. et al., Novel gene transfer into the fertilized eggs for goldfish (*Carassium auratus* L. 1785), Z. Angew. Ichthyol., 1985, 1: 31.
2. Zhu, Z., Xu, K., Li, G. et al., Biological effects of human growth hormone gene microinjected into the fertilized genes of loach Misgurnus anguillicaudatus (Cantor), Kexue Tongbao, 1986, 31(14): 988.
3. Dunham, R. A., Eash, J., Askins, J. et al., Transfer of the metallothioneinhuman growth hormone gene into channel catfish, Trans. Amer. Fish. Soc., 1987, 116: 87.
4. Penman, D. J., Beeching, A. J., Penn, S. et al., Factors affecting survival and integration following microinjection of novel DNA into rainbow trout eggs, Aquaculture, 1990, 85: 35.
5. Fletcher, G. L., Shears, M. A., King, M. J. et al., Evidence for antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*), Can. J. Fish. Aqaut. Sci., 1988, 45: 352.
6. Shears, M. A., Fletcher, G. L., Hew, C. L. et al., Transfer, expression and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*), Mol. Mar. Biol. Biotechnol., 1991, 1(1): 58.
7. Zhu, Z., Xu, K., Xie, Y. et al., A model of transgenic fish, Science in China, Ser. B (in Chinese), 1989(2): 147.
8. Zhu, Z., Growth hormone gene and the transgenic fish, in Agricultural Biotechnology (eds. You, C. B., Chen, Z. L.), Beijing: China Science and Technology Press, 1992, 106.
9. Westhusin, M., From mighty mice to mighty cows, Nature Genetics, 1997, 17: 4.
10. Wang, Z., Zhu, Z., Chromosomal *in situ* hybridization of Cyprinus carpio genomic DNA repetitive sequence CR1, Chinese Science Bulletin, 1999, 44(24): 2242.
11. Zeng, Z., Zhu, Z., Transgenes in F_4 pMThGH-transgenic red common carp (*Cyprinus carpio* L.) are highly polymorphic, Chinese Science Bulletin, 2001, 46(2): 143.

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