Nam and Kim #1 Tissue-specific expression of pFV4CAT in transgenic mud loach (*Misgurnus mizolepis*) germ line

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The transgene, pFV4CAT, containing CAT reporter gene regulated by carp β-actin promoter, was expressed in independent transgenic mud loach germ lines, determined by reverse transcriptase-PCR (RT-PCR) and enzyme-linked immunosorbant assay (ELISA). Expression of the transmitted transgene was found to be tissue-specific in F1 and F2 generations. Tissue specificity of the expression was dependent on each transgenic line with reproducible patterns. Liver and spleen did express the transgene more frequently than other tissues tested, and muscle and heart revealed the higher amount of CAT than other tissues, while testes showed the lowest expression level. The highest level of CAT expression in muscle from a transgenic F1 line was corresponding to 68-fold compared to the basal levels of controls.

Introduction

The generation of transgenic fish has been given much attention as a potential tool in the study of the developmental genetics with altered patterns of gene expression (Stuart *et al.*, 1990 ; Hackett, 1993 ; Iyengar *et al.*, 1996), and many studies on transgene expression have been reported in transgenic fish. However, most transgene expressions have been shown to be transient except only a few reports (Stuart *et al.*, 1990 ; Bayer and Campos-Oretea, 1992 ; Lin *et al.*, 1994 ; Gibbs *et al.*, 1994 ; Alam *et al.*, 1996 ; Kinoshita *et al.*, 1996). Even if transmitted expression, the expression assays in most works have based their claims on transmitted expression examined with early embryos or larvae. Therefore, the study on the patterns of transmitted expression in adult transgenic fish has still remained insufficient (Iyengar *et al.*, 1996).

Mud loach, *Misgurnus mizolepis*, an important fish both for food and ceremonial works by Buddhists in Korea (Kim *et al.*, 1994), has attracted attentions to fulfill many requirements as a

potential model material for transgenic study (Nam *et al.*, 1996). We have already reported that microinjected pFV4CAT construct consisting of carp β-actin promoter/enhancer and CAT reporter gene, could effectively express the CAT protein in Fo mud loach, based on the immunohistochemical in situ analysis using anti-CAT (Nam and Kim, 1997).

The object of this study is to produce the transgenic line which could stably transmit the foreign gene, pFV4CAT to their subsequent generations, and also to examine the patterns of transgene expression in different tissues of transgenic mud loach lines.

Materials and methods

Fish and gamete collection

Eggs and sperm were obtained from 4-year-old mud loach, *Misgurnus mizolepis*, which maintained at Fish Genetic Engineering Laboratory, Department of Aquaculture, Pukyong National University using intraperitoneal injections of HCG as described by Kim *et al.* (1995).

Plasmid

Plasmid (pFV4CAT) for microinjection containing bacterial CAT gene and carp β-actin promoter and regulatory region was kindly provided by Dr. Hackett, University of Minnesota, USA. Plasmid DNA was concentrated to 50μg/ml in injection buffer (1 mM Tris pH 8.0 ; 0.1 mM EDTA, pH 8.0 ; 0.05 % phenol red) by ethanol precipitation, and then used for microinjection.

Generation of transgenic mud loach

One-celled embryos were microinjected into the center of blastomere by microinjector whose needle had a diameter of about 5μm. After microinjections, embryos were transferred to a 25 ℃ incubator until hatch. Five microinjected groups were produced from different crosses. After yolk sac absorption, the larvae developed from microinjected eggs were pooled, and grown as described by Kim *et al.* (1994). At 2 months of age, putative transgenic individuals were screened by PCR of DNA obtained from fin or blood, and grown for further analysis.

Transmission of pFV4CAT to F1 and F2

Transgenic Fo were artificially spawned as described above, and crossed with non-transgenic fish in order to produce F1 progenies. Transmission frequencies of pFV4CAT were evaluated by PCR analysis of randomly taken 54 F1 individuals from each transgenic line. F2 offsprings also produced from each selected F1 group by crossing to non-transgenics, and the transmission frequencies were examined as like as F1 offsprings.

Analysis of transgene

DNA was isolated from whole body of hatched larva or from caudal fin by conventional SDS/proteinase K method or from whole blood and testis of adult fish by rapid method developed in our laboratory (Patent no. 08/534,531, USA). PCR reaction mixture contained 20 mM Tris (pH 8.3), 1.5 mM $MgCl_2$,

25 mM KCl, 100μg/ml gelatin, 20 pmoles each PCR primer, 50μM each dNTPs, 2.5 U Taq DNA polymerase (Perkin Elmer Co.), and about 0.2μg of template DNA. The reaction was carried out at 94℃ for 1 min, 60℃ for 30 sec, 72℃ for 1 min for 30 cycles with 2-min initial 94℃ denaturation step. Two primers (FVC-1 and FVC-2) were selected to amplify the fragment of CAT sequence. Sequences of FVC-1 and FVC-2 were 5' CTATAACCAGACC GTTCAGC3' and 5' CGCCCCGCCCTGCCACTCATCGCAG3', respectively.

Ten μg of genomic DNA from blood of F1 was used for genomic Southern blot analysis. DNAs were digested with Dra II and electrophoresed on a 0.7% agarose gel. The gel was de-purinated, denaturated and neutralized as described by manufacturer's recommendations of non-isotopic labeling and detection kit (BM Co., Germany). Membranes were hybridized with a probe, linearized 11-digoxy-dUTP labeled pFV4CAT.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Several tissues, such as testis, heart, intestine, liver, muscle and spleen from 5-month-old Southern-positive F1 fish from each transgenic line were subjected to RT-PCR. One μg of total RNA extracted from each tissue was reverse transcribed (Takara Shuzo Co., Ltd., Japan) as described in manufacturer's protocol. PCR primers used in RT-PCR were same as those used in screening transgenic individuals.

Enzyme-linked immunosorbant assay of CAT

Enzyme-linked immunosorbant assay (ELISA) was performed to determine the level of CAT expression in different tissues of transgenic mud loach using CAT ELISA Kit (5Prime-3Prime, Inc., USA). Tissues from F1 and F2 transgenic fish were surgically removed and homogenized in extraction buffer (0.25 M Tris-Cl pH. 7.8 containing 1 mM phenylmethylsulfonylfluoride). Preparation of samples, antibodies, washing, color development and calculation were done as described in manufacturer's instructions. The concentrations of CAT protein in different tissue extract were determined from the standard curve which was developed using standard CAT enzyme (5Prime-3Prime, Inc., USA).

Statistics

The differences of means in ELISA were assessed by t-test and/or ANOVA test at $P<0.05$.

Results

Generation of transgenic mud loach

Hatching success of microinjected eggs were ranged 48.5 to 78.2% which slightly lower than those of non-injected controls (52.4 to 89.1%). However, no differences in early survival rates up to yolk sac absorption between microinjected groups (78.6 to 88.5%) and non-microinjected controls (78.7 to 91.7%) were observed.

A random sample of 27 2-month-old putative transgenic individuals were chosen from each microinjected group, and their fin or blood DNAs were subjected to PCR analysis. Trans-

genic Fo individuals could be isolated by PCR as evidenced by the presence of expected size of PCR product amplified from the genomic DNA of putative transgenic individuals (Fig. 1). The incidence of pFV4CAT was different among each microinjected group, ranged from 9.3 to 27.8%.

Southern blot analysis

No positive signal of hybridization was detected in the genomic DNA from non-injected control fish. On the other hand, Southern blot analysis of transgenic F1 individuals (PCR-positive) revealed the presence of pFV4CAT insert, and high molecular weight band which might indicate the integration of the transgene into mud loach chromosomal DNA (Fig. 2).

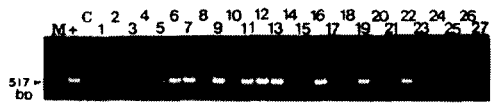

Fig. 1. Et-Br stained agarose gel showing the results of PCR screening transgenic fish. M, 1kb ladder ; +, pFV4CAT ; C, non-injected fish ; 1~27, microinjected fish.

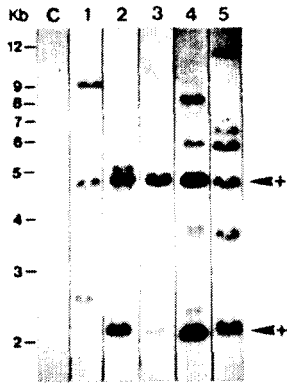

Fig. 2. Genomic southern blot analysis of microinjected and non-injected fish. DNAs were completely digested with Dra II, electrophoresed in 0.7% TAE agarose gel, transferred to nylon membrane, and hybridized with 11-digoxy-dUTP labeled linearized pFV4CAT. C, non-injected fish ; lane 1-5, PCR positive F1 fish. Arrow (+)s indicate the sizes of two fragments yielded by Dra II digestion of pFV4CAT.

Germ line transmission of transgene

Approximately, 12.5% (6/48) of the transgenic Fo individuals (fin or blood positive) could transmit transgene to their F1 offsprings. Transmission rates were significantly varied among Fo individuals, ranged from 5.5% to 33.4% However, most F1 individuals transferred their transgenic insert into F2 generation with expected Mendelian ratios (approximate 50%) (Table 1).

Expression of CAT mRNA in different tissues of transgenic F1 fish

The presence of CAT mRNA in different tissues, including testis, heart, intestine, liver, muscle and spleen of adult transgenics, were determined by reverse transcriptase-PCR (RT-PCR). RT-PCR produced expected size of PCR products from transgenic F1 individuals, which was exactly matched to that from pFV4CAT (Fig. 3). No positive signal was detected in any tissues from non-transgenic fish. The pres-

Table 1 Germ line transmission of pFV4CAT determined by PCR analysis in mud loach, Misgurnus mizolepis

Fo	% transgenics	
	F1	F2
FCat#1	7.4 (4/54)	48.1 (26/54)
FCat#2	14.8 (8/54)	46.3 (25/54)
FCat#3	33.4 (18/54)	50.0 (27/54)
FCat#4	18.5 (10/54)	53.7 (29/54)
FCat#5	18.5 (10/54)	48.1 (26/54)
FCat#6	5.5 (3/54)	31.5 (17/54)

*Figures in parenthesis are no. of transgenics/no. of analyzed fish.

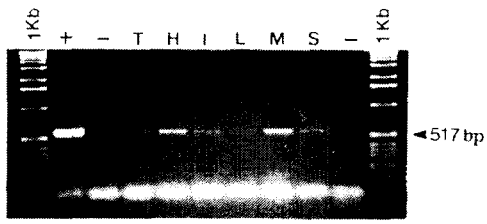

Fig. 3. Detection of CAT mRNA in different tissues of a 5-month-old F1 transgenic mud loach, based on the RT-PCR. +, pFV4CAT ; −, negative blank, T, testis ; H, heart ; I, intestine ; L, liver ; M, muscle ; and S, spleen.

ence of CAT mRNA in different tissues of adult transgenic F1 fish was different depending on transgenic lines. The presence of CAT mRNA was detected in liver and spleen more frequently than in other tissues (Table 2).

Levels of CAT expression in different tissues of transgenic F1 and F2 fish

The amount of expressed CAT protein was also quitely varied among transgenic lines, based on the ELISA. Tissues from non-transgenic control fish showed little negligible basal level of CAT signal. Muscle and heart revealed the higher amount of CAT than other tissues, while testis showed the lowest expression level. The highest level of CAT expression was observed in muscle from F1 of FCat#4 line, that was corresponding to approximately 68-fold, compared to basal value of muscle from non-transgenic (Fig. 4).

The level of expression in F2 was lower than F1 in most cases. The reduced amount of expression in F2 varied among transgenic lines and different tissues. However, FCat#4 (except in muscle), FCat#5 and FCat#6 lines showed the stable transmitted expression in F2 without losing the activity (Fig. 4).

Table 2 Expression of pFV4CAT in different tissue from F1 transgenic mud loaches, Misgurnus mizolepis, determined by RT-PCR

Transgenic line	Tissue					
	Testis	Heart	Intestine	Liver	Muscle	Spleen
FCat#1	+	+	+	+	+	+
FCat#2	+	+	+	+	+	+
FCat#3	−	−	−	+	−	−
FCat#4	−	+	+	+	+	+
FCat#5	−	−	−	−	−	+
FCat#6	−	−	−	+	−	+

+, RT-PCR positive
−, RT-PCR negative

Discussion

The expression of integrated foreign gene was evidenced by Southern blot hybridization. Southern blot analysis showed both the expected size of transgenic insert and presence of junction fragments to host

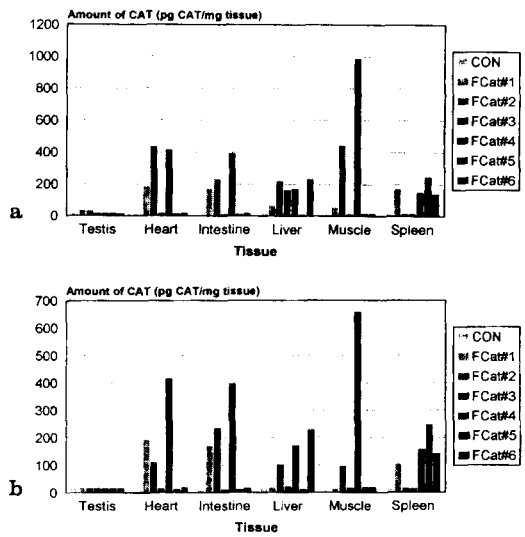

Fig. 4. Level of CAT expression (pg CAT/mg tissue) in different tissues of adult F1 (a) and F2 (b) transgenic mud loach, based on the CAT-ELISA.

chromosomes. Additional minor bands in Southern analysis presumably reflect either multiple integration sites, or undefined rearrangements of foreign DNA as suggested by Wang *et al.*, (1995).

Transgene construct, pFV4CAT could express foreign CAT protein in F1 and F2 generation of transgenic mud loach. The presence of CAT mRNA confirmed by RT-PCR was different in tissues depending on each transgenic lines. And the levels of transgene expression in different tissues, based on the ELISA were also different among transgenic lines. These results were similar to transgenic carp, where the tissues expressing rainbow trout growth hormone cDNA regulated by RSVLTR promoter, were varied among transgenic lines and the amount of rtGHmRNA in tissues were also different, determined by RNA dot-blot assay (Chen *et al.*, 1993). Tissue-specific expression of pSUCAT in tissues of transgenic zebrafish have been also reported by Stuart *et al.* (1990). The line-specific differences in transgene expression may suggest distinct position effects indicating the integration at independent sites. One possible explanation for these variegated expression is that these patterns of transgene expression were resulted from somatic mutation (instability of transgenic insert) or mosaic gene activation or inactivation (Stuart *et al.*, 1990 ; Alam *et al.*, 1996).

A founder and one F1 fish did transfer the pFV4CAT construct to subsequent generation, with little or no expression of transgene. The inactivation of integrated transgene has been frequently found in transgenic fish (Iyengar *et al.*, 1996) as well as in other vertebrates (Dorer, 1997). Silence of transgene and non-uniform expression among transgenic lines observed in this study may be explained as position effects, heterochromatin formation or DNA methylation of transgene (Gibbs *et al.*, 1994 ; Alam *et al*, 1996 ; Iyengar *et al.*, 1996 ; Koetsier *et al.*, 1996).

Generally, level of expression in F2 was reduced compared to F1, although two transgenic lines maintained the stable level of expression. Reduction or lack of expression upon transmission to subsequent generations has been observed in several transgenic fish (Stuart *et al.*, 1988 ; 1990 ; Culp *et al.*, 1991 ; Wang *et al.*, 1995 ; Alam *et al.*, 1996). However, recent work on transgenic medaka conducted by Kinoshita *et al.* (1996) demonstrated that the stable expression of transgene could be transmitted in homozygous state even in the F6 generation.

Further researches will be made to examine if stable expression with tissue-specific patterns in present study could be maintained to further subsequent generations.

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미꾸라지(**Misgurnus mizolepis**)에서 **pFV4CAT**의 조직 특이적 발현

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외래유전자, pFV4CAT이 이식된 transgenic 미꾸라지 계통 F1 및 F2를 대상으로 조직별 외래 유전자의 발현을 조사하였다. Transgenic F1에서 pFV4CAT의 mRNA 합성 여부를 조사하기 위해 정소(testis), 간(liver), 근육(muscle), 비장(spleen) 및 심장(heart) 조직을 RT-PCR로 분석한 결과, 조직별 mRNA 존재 여부는 F1 계통간 큰 차이를 나타내었으며, 다른 조직들에 비해 간(liver)과 비장(spleen)에서 보다 빈번히 발현하는 경향을 나타내었다. 외래 유전자에 의해 합성된 CAT 단백질을 ELISA로 정량화한 결과, 조직별 및 transgenic 계통별 다양한 차이가 있었으며, 다른 조직에 비해 근육과 심장에서 가장 높은 수준으로 발현하는 것으로 나타나 F1 한 계통의 근육에서, 대조군 수치의 최고 68배에 해당하는 CAT 발현이 관찰되었다. 반면 정소에서 가장 낮은 외래 유전자의 발현이 모든 transgenic line에서 관찰되었다.