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Transgenesis in Fish: Indian Endeavour and Achievement

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The first Indian transgenic fish was generated in 1991 using borrowed constructs from foreign sources. To construct transformation vectors for the indigenous fishes, growth hormone genes of rohu (r-GH), *Labeo rohita* and catfish, *Heteropneustes fossilis* were isolated, cloned and sequenced; their fidelity was confirmed in prokaryotic and eukaryotic systems. A vector was constructed with grass carp b-actin promoter driving the expression of r-GH. Rohu eggs are large, fragile and swell 2~3 times, when fertilized. Hence they were amenable only for electroporated sperm-mediated gene transfer. Accordingly, the sperm electroporation technique was standardized to ensure 25% hatchling survival and 37% presumptive transgenics without suffering any deformity. Southern analysis confirmed genomic integration in 15% of the tested individuals (Ti) belonging to family lines 2 and 3; another 25% of the juveniles (Te) were also proved transgenic but with the transgene persisting extrachromosomally for longer than 1 to 2 years, perhaps due to the presence of replicon in the vector. Transgenics belonging to different family lines grew 6~8 times faster than the respective controls. Difference in growth trends of Ti and Te within a family line was not significant. In the Ti family 3 remarkable growth acceleration was sustained for a period longer than 36 weeks but in those of family 2, it gradually decreased. All transgenic fishes including the rohu converted the food at a significantly higher efficiency. Barring the transgenic mudloach, all the other transgenic fishes consumed food at significantly reduced rate.

Keywords: Cloning and sequencing growth hormone (GH) gene, Transformation vectors, Gene transfer by electroporated sperm, Genomic integration, Fast growing transgenic rohu, Reduced feeding rate, Enhanced conversion efficiency

Introduction

Selective breeding is a proven method for strain improvement of farm animals but suffers from the following: (i) requires longer time, (ii) may transmit unwanted traits due to mixing of whole genome of the parents and (iii) prevents transfer of genes from one species to another due to the interspecific biological barrier (Pandian, 2001). Transgenesis, however, allows the introduction of novel gene or augmentation of a specific trait by just transferring tailored copies of gene from the same or other species. Transgenesis may be defined as the introduction of exogenous DNA into the host genome resulting in its stable maintenance, transmission and expression. The limited availability of piscine genes and promoters, and the restricted scope for cytoplasmic introduction of the transgene are the major hurdles in generation of useful transgenic fish (Pandian and Marian, 1994).

Thanks to the recent developments in molecular biology, more than 8,500 genes and cDNA sequences of piscine (commercial fish only) origin have been isolated, characterized and cloned. Of these, 101 constitute commercially important genes belonging to the somatotropin family and only 44 of them are growth hormone (GH) sequences; less than a dozen of these sequences are inserted into appropriate vectors and are ready for gene transfer studies (Table 1). A survey of these vectors shows that the available vectors/constructs are mostly for the economically important fish of the developed countries. With the kind of patenting and intellectual property rights system prevailing in the international scenario, it has become a necessity for the developing countries, which depend on fisheries as an important food source, to develop all-fish gene constructs for growth enhancement in fish species that are economically important to them. Secondly, our present knowledge on structure and function of eukaryotic gene has shown the need for introns, enhancer, boundary and locus control regions. A variety of genes and other sequences originating from bacteria to mammals have been used for want of suitable genes of piscine origin. Due to inappropriate splicing and translations, achieving good expression of heterologous gene/cDNA remained a problem. In India, research in

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Table 1. Available growth hormone genes/DNA sequences of piscine origin (from Biswas et al., 2001).

Somatotropin hormone family	108
Sequences in vector form	20
Growth hormone (GH) sequences	44
GH sequences in vector form	9
All-fish GH in vector form	6
GH sequences of piscine origin from India	2

transgenic fish was initiated at the Madurai Kamaraj University with borrowed constructs from foreign scientists (Pandian et al., 1991). In 1998, a couple of vectors were constructed using again borrowed growth hormone (GH) gene sequences (Sheela et al., 1998). Therefore, our first task was to isolate, clone and sequence the growth hormone (GH) gene of a couple of economically important Indian fishes, *Heteropneustus fossilis* (Anathy et al., 2001) and *Labeo rohita* (Venugopal et al., 2002a) and to construct expression vectors including GH cDNA and b-actin promoter of grass carp (Anathy, 2002). This presentation is however limited to *L. rohita* alone.

Indigenous Transfer Vectors

The cDNA clones encoding for growth hormone (GH) were isolated from the pituitary. Partial GH cDNA (3end) was amplified by RT-PCR and used as probe to screen the cDNA library. Full-length GH-specific cDNA clone (1180 bp) was isolated and sequenced. The sequence contains 48bp 5 noncoding region followed by an ORF of 621 bp and a 3 noncoding region of 521bp (Venugopal et al., 2002a). The peptide shares about 90% identity with the GH of *Cyprinus carpio* (Linn.) and >84% identity with GH sequences of other cyprinids (Venugopal et al., 2002b). The GH-encoding open reading frame (ORF) of rohu has been cloned into expression vectors, and GH protein has been over-expressed in *Escherichia coli* and purified as a soluble protein (Fig. 1). The GH cDNA was also cloned into a bicistronic vector with EGFP; injection of *in vitro* transcribed GH-EGFP mRNA into zebrafish embryo has resulted in EGFP expression confirming the cloned GH cDNA is functional in fish (Fig. 2) and the IRES element can be effectively used in fish for bicistronic expression of foreign genes (Venugopal et al., 2002a). Subsequently, a vector was constructed with grass carp b-actin promoter driving the expression of r-GH (Fig. 3) (Anathy, 2002). The b-actin promoter is known to express better than several homologous and heterologous promoters in fish cells (Friedenreich and Schartl, 1990) as it contains a typical CAAT box, a TATA box, and an evolutionarily conserved CC(AT)6GG

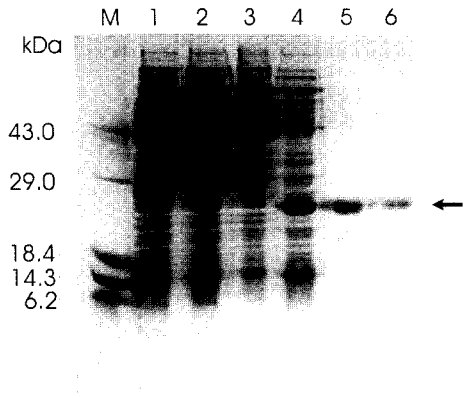

Fig. 1. Expression of r-GH in *E. coli*. SDS-PAGE analysis of proteins; lane M, Protein molecular weight marker. The figure shows the profile of total crude protein extract of pQ30 vector containing *E. coli* cells before (1) and after IPTG induction (2) and the cells harboring the vector with GH insert before (3) and after the IPTG induction (4). Purified protein fractions of 100 mM (5) and 250 mM (6) imidazole elution are also shown (from Venugopal et al., 2002a).

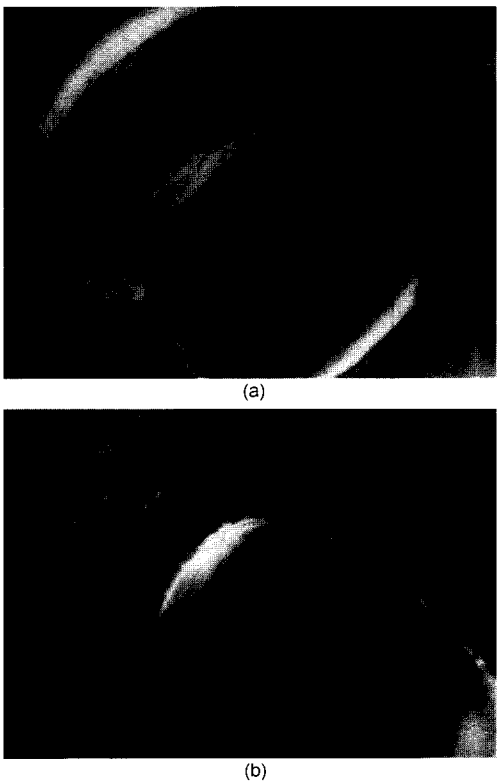

Fig. 2. Expression of r-GH in zebrafish. The *in vitro* transcribed RNA was mixed with injection solution (0.1M Tris-Hcl, 0.24% phenol Red, pH 7.6) to a final concentration of ~100 ng/µl. About 6 nl of bi- cistronic r-GH-IRES-EGFP RNA was injected into the single-celled zebrafish embryos. The embryos were then incubated at 28°C and EGFP expression was monitored from 6 hrs after injection. Figure shows the embryos that were 11 (a) and 18 (b) hr after injection (from Venugopal et al., 2002a).

unit, termed as CarG motif (SRE). The vector pgcb-r-GH-IRES-EGFP contains homologous rohu GH cDNA, an Internal Ribo-

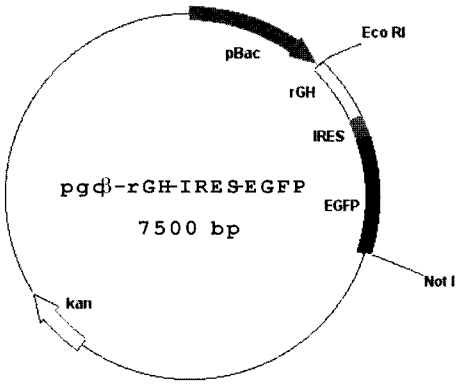

Fig. 3. The gene transfer vector containing grass carp β-actin promoter, endogenous rohu r-GH cDNA, IRES and EGFP reporter (from Anathy, 2002).

somal Entry Site (IRES) element downstream to the r-GH cDNA and the Enhanced Green Fluorescent Protein (EGFP) reporter gene to facilitate the bi-cistronic expression. To ensure sustainable multiplication of the vector, the plasmid also contains replicon of eukaryotic origin (Anathy, 2002). Incidentally, the origin of replicon is essential for the perpetuation of any DNA *in vivo* (Darnell et al., 1986) and the plasmids that lack the origin of replication are diluted out of their host cell in course of time. Unfortunately, several researchers have not realized the importance of this and have not included eukaryotic replicon in their vectors (e.g. Dunham et al., 1987). Others, who have made vectors exclusively for fish, have not emphasized its importance (e.g. Liu et al., 1990).

Transfer of Transgene

Microinjection is a widely used efficient gene transfer method in fish. It is shown to ensure genomic/extrachromosomal persistence of transgene in 20 to 90% of fishes at different ages ranging from 3 weeks to 2 years. Table 2 lists problems encountered in microinjection and adopted strategies to solve/minimize the encountered problems (Venugopal, 2002). Microinjection is also time-consuming, laborious, species-specific and in some cases technically demanding. Besides, the eggs of tropical fish such as rohu commence cell division in less than 30 minutes after fertilization and have only a shorter time window for microinjection. For this reason, Alok et al. (1995), who have microinjected fertilized eggs of rohu with plasmid containing human growth hormone and murine metallothionein promoter, could obtain only 5 fry, which too succumbed within 90 days.

In fish electroporation is the next preferred method for gene transfer. The technique of transferring the transgene into eggs of rohu using electroporation encountered an unusual problem. As the fertilized rohu eggs are large (1.8±0.1 mm), the largest cuvette of the Gene pulser II (0.4 cm gap, catl no: 165-2088 of Biorad) can accommodate a maximum of 150 eggs and buffer. Moreover, the eggs of rohu are fragile and swell 2~3 times within 15~30 minutes following fertilization and attain a size of 2.5 to 5.0 mm in diameter. When ~150 eggs are accommodated in the cuvette for electroporation, ≥ 95% of the eggs suffer mortality (even at 150 Volt/cm) due to swelling. Hence, only about 50 eggs can be electroporated at a given time.

This unusual situation led to the development of a protocol for sperm-mediated gene transfer into rohu eggs. An earlier study by Chourrout and Perrot (1992) on rainbow trout to generate transgenic fish by sperm-mediated transfer has

Table 2. Problems encountered and strategies adopted to transfer of gene in fish eggs by microinjection (from Venugopal, 2002)

Problem	Strategy	Species
Invisible nucleus	Centrifugation and staining with vital dye (yet unsuccessful)	Salmon
	Dechorionation and staining with fluorescent dye	Goldfish
	Cytoplasmic microinjection	Common carp
	Microinjection into germinal vesicle	Medaka
Hard chorion	Surgical removal of ovary and *in vitro* oocyte culture until microinjection	Medaka
	Use of cold water and Ringers to slow down chorion hardening	Medaka
	Injection through micropyle	Channel catfish
	Cytoplasmic microinjection with trypsin (0.2-0.25%) digestion of chorion	Goldfish
	Cytoplasmic microinjection with pronase (500 µg/ml) digestion the chorion	Zebrafish
	Cytoplasmic microinjection after manual dechorionation	Atlantic salmon
	Drilling the chorion	Rainbow trout
	Soaking eggs with 0.5 mM glutathion	Rainbow trout
Difficulty in locating micropyle	Injection through chorion	Common carp

Table 3. Persistence of transgene in sperm-mediated DNA transfer in fishes. Persistence confirmed by: sl- slot blot analysis; E- enzyme assay; MA-Microscopic autoradiography; P- PCR analysis; R- RT-PCR analysis; S- Southern blot analysis (from Anathy, 2002)

Species	Vector	Age of fish	Transgene persistence	
			(%)	Confirmed by
Without electroporation				
Clarius gariepinus	PRSVlacZ	10 days	0	sl
Cyprinis carpio	pHSVtkneo	1-2 weeks	0	sl
Oncorhynchus mykiss	pRSVCAT	7 weeks	0	sl
Danio rerio	pCMVCAT	1 week	38	sl, E
D. rerio	pMTL	2 weeks	7	P, MA
With electroporation				
C. gariepinus	PRSVlacZ	10 days	3	sl, E
Oreochromis niloticus	pGMH4CAT	1 month	3	sl
C. carpio	PRSVlacZ	2 weeks	3	sl
O. tshawytscha	pCMVlacZ	Fry	2	sl
D. rerio	pMTL	2 weeks	12.5-14.5	sl
Labeo rohita	pRSVrtGH	2 weeks	14	sl
Cirrhina mrigala	pRSVrtGH	2 weeks	23	sl
Catla catla	pRSVrtGH	2 weeks	13	sl
Misgurnus anguillicaudatus	pAFPsGH	2 weeks	37	P, MA
L. rohita	pCMV-r-GH-IRES-EGFP	60 weeks	25	sl, S, R
L. rohita	pgcβ-r-GH-IRES-EGFP	36 weeks	25	sl, S, R

invariably failed to detect transgene. Although Khoo et al. (1992) have successfully transferred the plasmid containing CAT gene into zebrafish eggs through sperm-mediated transfer; it may be indicated that barring Tsai et al. (1995), previous authors have invariably attempted to transfer one or the other reporter construct through incubation or electroporation of sperm (Table 3). Almost all of them confirmed the presence of the transgene in fry, whose age was less than 2 weeks, and invariably the confirmation was made by slot blot analysis alone. Venugopal et al. (1998) standardized the various parameters for sperm electroporation in rohu and achieved a higher survival (70%) and gene transfer frequency (25%) even at the lowest field strength clearly indicating that their protocol is more perfect than that adopted by Tsai et al. (1995). Despite achieving 37% gene transfer frequency, Tsai et al. (1995) encountered >30% deformity in the generated transgenics.

Destiny of the Transgene

Both slot and Southern analyses were made to trace the destiny of the transgene in 3 different family lines of the presumptive transgenic rohu. In family lines 1, 2 and 3 presence of the transgene was confirmed in 25% of the tested fry (Table 3). Southern blot also showed its intact but extrachromosomal persistence in fairly large number of tested (25~43%) juveniles at the age of 30 and 60 weeks in family 1 and 14 and 35 weeks in family 2 and 3 (Fig. 4c). Our studies have clearly shown that despite decreases in copy number, the transgene continued to persist extrachromosomally in these juveniles of all the 3 family lines until the age of 1 or 2 years. It is not clear, whether the presence of the replicon is responsible for the observed intact but extrachromosomal persistence of the transgene for such a long duration. For instance, Nam et al. (1999) recorded the extrachromosomal persistence and transmission of the transgene (pFV4aCAT) upto F_3 progenies of the mud loach *Misgurnus mizolepis*; earlier Stuart et al. (1988) have reported the transmission of extrachromosomally persisting transgene in the zebrafish *Danio rerio*. Hence the transgene not only persists extrachromosomally for long periods but also is transmitted to progenies.

At the age of 14, a few other juveniles belonging to families 2 and 3 could be individually identified; they could also provide adequate fin tissues for analysis. Surprisingly, 3 out of 5 tested rohu (Ti) of each family gave clear signals for genomic integration (Fig. 4a,b). The integrated copies hybridized to b-actin or r-GH only but not to EGFP or to the backbone of the vector, indicating that only the b-actin-r-GH fragment of the vector was integrated into the genome of the transgenic rohu.

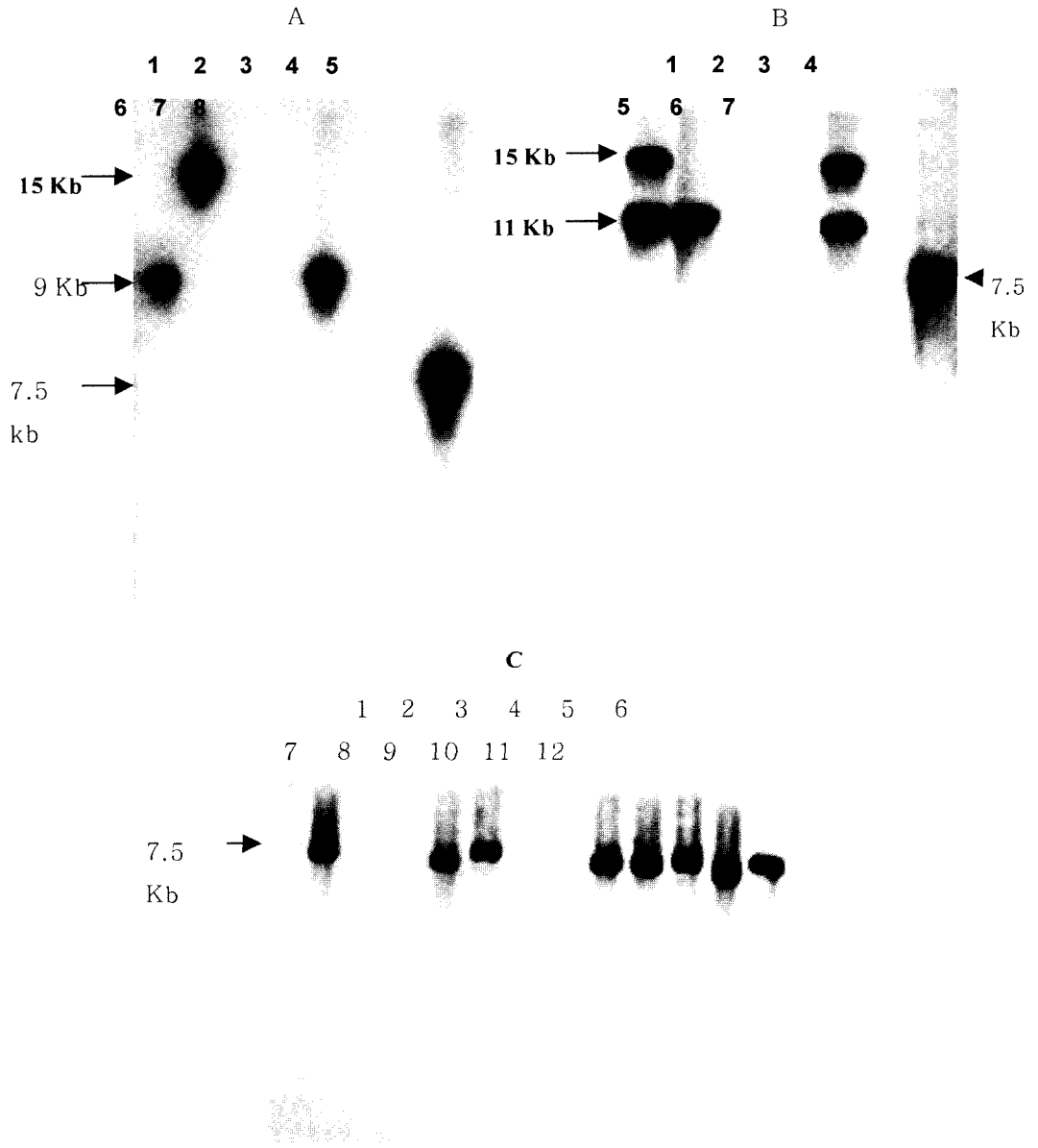

Fig. 4. Southern hybridization of the rohu juveniles. Genomic DNA from the fin (extracted at the 14th week) digested with *Eco* R I probed with grass carp b-actin promoter. Positive signals at 9, 11, and 15 Kb regions (indicated by arrow) suggest integration in the identified individuals belonging to family 2 (#12, #13, #15) and 3 (#14, #15, #20) in panels A and B, respectievly. C shows individuals harboring extrachromosomal copies. (from Anathy, 2002).

Growth of Transgenics

Expression of the integrated/persisting transgene was confirmed by RT-PCR analysis made on identified Ti and Te individuals belonging to families 2 and 3. However, this presentation highlights the ectopic expression of GH in terms of growth from the fortnightly measurements of transgenics and controls.

Less than 0.1% of the hatchlings, that were generated from rohu eggs fertilized by electroporated sperm survived. However, none of the hatchlings ever suffered from any deformity (Fig. 5). Almost all the experimental juveniles belonging to the 3 different family lines grew significantly faster than the respective control siblings (Fig. 6). For instance, the experimental progenies belonging to the family 2 grew upto 26 g at the age of 36th week. Notably, the progenies of family 3 grew even faster and achieved the largest body weight of 49 g (Fig. 5). The individual size variation was limited within the experimental as well as controls; at the age of 36th week variations were within 22~26 and 40~49 g for family 2 and 3, respectively (Fig. 6).

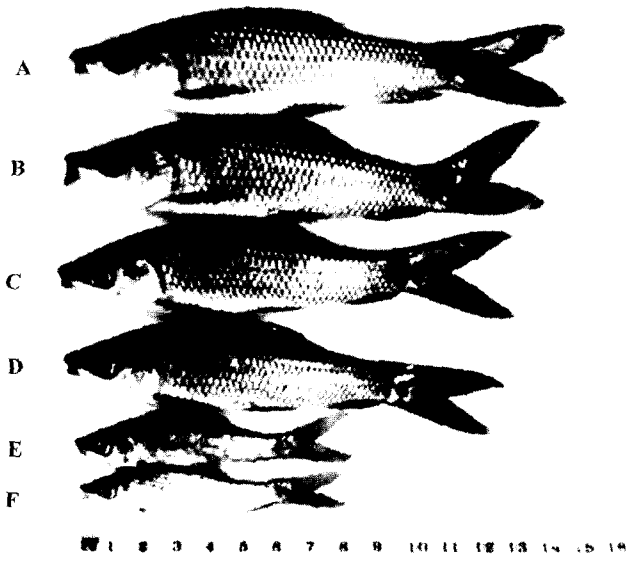

Fig. 5. Transgenic rohu (36-week old) of family 2 and 3. A=Ti rohu of family 3 (49 g); B=Te rohu of family 3 (46 g); C=Ti rohu of family 2 (26 g); D=Te rohu of family 2 (24 g); E & F=Control siblings of family 3 (7 g) and 2 (6 g) (from Anathy, 2002).

Growth trends of identified (Ti and Te) individuals belonging to these 3 family lines are also shown in the window (Fig. 6). These trends confirm each other and show that the transgenics were growing 4~7 times faster than their respective control siblings. Remarkably, the maximum body weight attained at the age of 36 weeks by the transgenic rohu was 23, 24, 26, 46 and 49 g for the Te of family 1, Te of family 2, Ti of family 2, Te of family 3 and Ti of family 3, respectively. From these observations, two generalizations can be made: (i) Transgenics belonging to family 3 were growing about 2 times faster than those belonging to family 1 and 2, and (ii) The difference observed between Te and Ti transgenic rohu belonging to a family is not significant. Therefore, there is a need for selecting even among the transgenics, a family line with the potential for super fast growth (see also Nam et al., 2001).

From the 14th week, growth of identified Ti rohu of family 2 (2#12, 2#13, 2#15) and family 3 (3#14, 3#15, 3#20) was individually traced upto 36th week. Remarkably, growth of the transgenic #14 was the fastest; at the age of 36th week, it attained a body weight of 49 g, in comparison to 7 g weight attained by the control, i.e. its growth acceleration was nearly 7 times faster. Analysis of values obtained for the mean growth acceleration of the identified Ti rohu of family 2 and 3 indicated significant difference between them. In family 2, the acceleration decreased from 7 times at the age of 14th week to 4.4 times at the age of 36th week (Fig. 7a). Conversely, there was significant difference between the trends for growth acceleration vs age of the 3 identified rohu belonging to family 3, indicating that even among the transgenics belonging to the same family, the level of acceleration differed significantly. Secondly, the growth acceleration in 3#14, 3#15 and 3#20 remained stable at the levels of 5.8, 5.5 and 4.9 times, respectively (Fig. 7b). Briefly, these Ti individuals were characterized by i) the sustenance of growth acceleration at the respective constant levels from 14th to 36th week and ii) the significant difference in the levels of growth acceleration. On the other hand, exactly the reverse was true for the identified individuals of family 2.

Levels and trends obtained for growth acceleration in

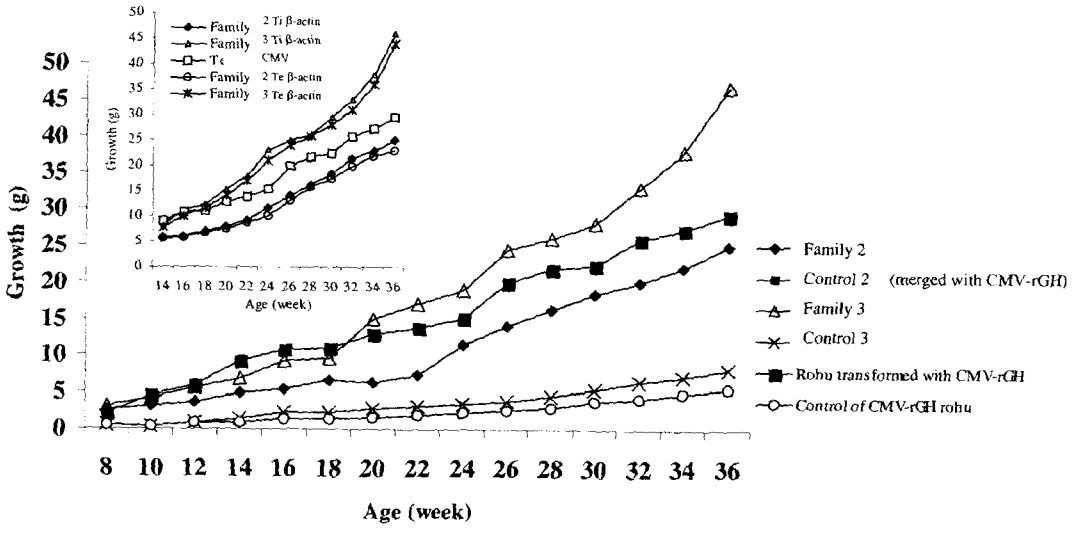

Fig. 6. Growth trends of transgenic rohu belonged to family lines 1, 2 and 3. Inserted window depicts comparative growth trends of identified Ti and Te rohu of these family lines. The transgenics carry a vector containing r-GH fused to CMV promoter (family line 1) or b-actin (family line 2,3) (from Venugopal et al., 2002c).

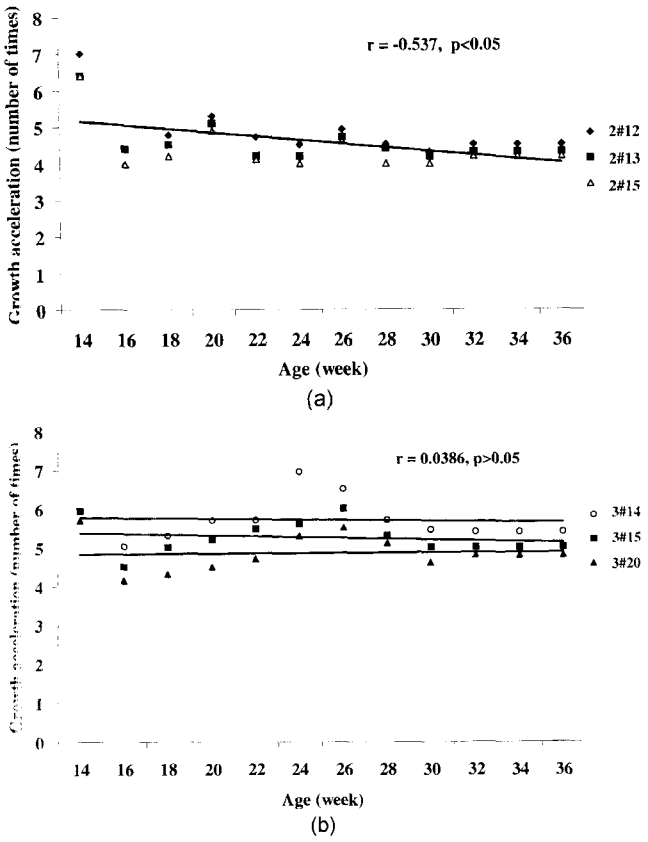

Fig. 7. (a) Growth acceleration in Ti rohu (family 2). Each value represents one individual (from Venugopal et al., 2002c), (b) Growth acceleration in Ti rohu (family 3). Each value represent one individual (from Venugopal et al., 2002c).

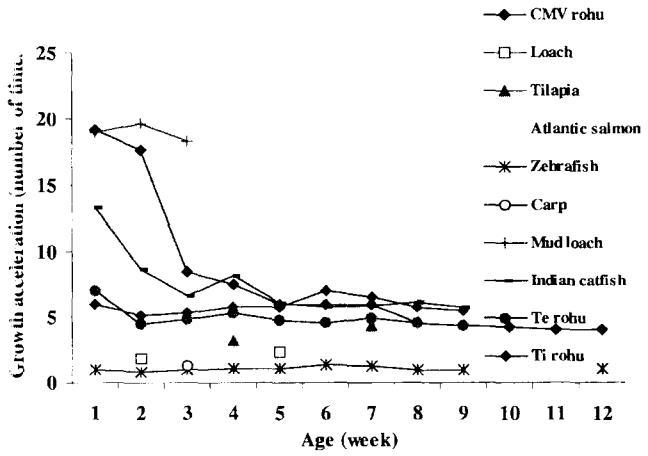

Fig. 8. Trends obtained for growth acceleration in selected transgenic fishes. (from Venugopal et al., 2002c).

selected transgenic fishes (Fig. 8) suggest 3 different trends: (i) stable growth acceleration at different levels: 1~2 times (e.g. *D. rerio*: Pandian et al., 1991), 4-6 times (*H. fossilis*: Sheela et al., 1999; rohu family line 3: Anathy, 2002), and ~20 times (*M. mizolepis:* Nam et al., 2001); (ii) an increasing trend: e.g. from 1.8 to 2.4 times (*M. anguillicaudatus*: Tsai et al., 1995) and 2.8 to 5 times (*Salmo salar*: Du et al., 1992) and (iii) a steep fall from about 11 times and subsequent stabilization at about 5 times (rohu: Venugopal et al., 2002c). Remarkably, all these transgenics display reliable and measurable growth acceleration.

Table 4. Weight gain, food intake and food conversion efficiency (FCE) of 8-month old Ti (29.3 g) and their control (5.04 g) siblings during the 30-day feeding experiment. Values followed by different superscripts differ significantly (t test; P<0.05). (from Anathy, 2002)

Parameter	Control	Transgenic
Feeding rate (% body weight/day)	2.5 ± 0.12^{aa}	1.6 ± 0.06^{ab}
FCE (%)	72.6 ± 8.66^{aa}	159 ± 25^{ab}

Feeding Rate and Conversion Efficiency

Feeding rate and conversion efficiency are important parameters to be considered to cut down the cost of production in aquaculture enterprise. To estimate the rate at which food is consumed and efficiency with which the consumed food is converted into body own substance, the 8-month old Ti rohu individuals of the family 3 were subjected to feeding trials. Feeding rate of the Ti was 1.6% body weight/day, in comparison to 2.5% body weight/day for the control (Table 4). Thus, the transgenics consumed the food at a significantly reduced rate, i.e. 60% of the control value. Not only the transgenics reduced the feeding rate but also converted the food more efficiently, i.e. the Ti converted food at 160% efficiency, while the control at 73% efficiency. Incidentaly, almost all the transgenic fishes are known to significantly enhance the food conversion efficiency. This is clearly an advantage to aquaculture.

While the transgenic mudloach maintained the feeding rate and growth acceleration at respective high levels (Nam et al., 2001), rohu and other transgenics reduce feeding rate and correspondingly suffer reduction in growth acceleration.

Acknowledgements

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