特別講演

座長：荒井克俊（北海道大学大学院水産科学研究院）

演者：Yoon Kwon NAM（大韓民国・釜慶大學校水産科学大学）
Abstract

Generation of valuable transgenic fish with desirable phenotypes has potential implications for food production as well as many other bio-industries. Transgenic engineering of fish genome would provide a quantum leap over traditional selection and breeding methods by offering the powerful means to increase the efficiency of farming practices with desired traits. With this purpose, research with a wide spectrum of success has been in progress for the past two decades to generate valuable transgenic fish strains. Transgenic manipulations have already made an important contribution in all disciplines fisheries sciences and aquaculture, however, commercial application of transgenic fish strains is still complicated by many scientific and social issues. Here this mini-review introduces briefly the historical background, principle, current status and perspective of transgenic manipulation in fish.

Introduction

Tremendous progress in genetic manipulations during last decades provides fisheries and aquatic sciences with new tools. Scientists became capable of modifying genome of complex organisms. The first genetically modified mice were obtained shortly thereafter, and we have moved so fast to a new era in which transgenic animals including fish should aid in solving many problems in fields such as food security, public health, livestock improvement and biomedical researches. Transgenic animal models also have played an important role in gaining new insights into the knowledge in many biological fields especially in molecular and developmental research.

Transgenic fish research has been underway for nearly 20 years. Since the mid-1980s when the first transgenic fish were successfully produced (Zhu et al., 1985), gene transfer studies have been conducted in over 35 fish species, including commercially important species. Main purposes for transgenic researches have been either (1) illumination of gene regulation of vertebrates or (2) the development of useful broodstock with enhanced performance in aquaculture. Numerous transgenic fish strains have been developed as experimental models for biomedical and developmental researches (Fetcho and Liu, 1998; Dooley and Zon, 2000; Goldman et al., 2001; Ward and Lieschke, 2002), since fish system has many attractive traits as vertebrate laboratory model organisms: use of fish model in certain research areas can significantly reduce the exploitation of mammals, decrease costs and speed the research process. Regarding the productivity enhancement in aquaculture, the growth improvement has been the most frequent target in transgenic researches. Currently, at least one US biotechnology company, AquaBounty Farms (Waltham, MA) is close to commercializing transgenic salmon engineered to acquire fast growth rate. Transgenic technologies will be integrated with not only other modern platform technologies but also genetic breeding programs for enhancing aquaculture production.
Fish as model material for transgenic research: advantages and limitations

Transgenic animals containing novel heterologous genetic information can provide many useful systems for addressing fundamental questions from a variety of biological studies (Iyengar et al., 1996). Transgenic fish can offer an alternative to previous mammalian models as vertebrate laboratory animals. Many fish species produce zygotes via external fertilization: eggs and milts can also be stripped from ripe fish, fertilized by hand and the resultants embryos reared to hatch, allowing us to literally visualize all stages of vertebrate development. Currently small aquarium fish species such as zebrafish and medaka have been given much attention as emerging vertebrate models for genomic and transgenic researches. Oviparous zebrafish have the advantage that their externally fertilized eggs can develop rapidly as transparent embryos: it is possible in zebrafish to visualize real-time imaging in gene expression and detailed morphogenetic movements as they transpire in a live, developing vertebrate embryo.

Besides these small aquarium fish, several fish species grown for commercial purposes with relatively large size have also been considered as candidate models system for transgenic researches. They are common carp (Cyprinus carpio), tilapia (Oreochromis niloticus) and rainbow trout (Oncorhynchus mykiss). The major advantageous merits of these species over small aquarium fish are high fecundity. Under controlled system, thousands of eggs can be easily obtained from a single female, indicating that large number of siblings with similar genetic background could be produced from a single mating pair. It has important implication in the large scale propagation of homogenous isogenic population with desired traits from a transgenic broodfish through genetic breeding program (Maclean et al., 2002). Another advantage is the easiness to obtain biological samples for biochemical and genomic assays: blood and tissue volumes are much increased when compared to those of small-sized model fish, which is important for engineering of transgenic bioreactors. On the other hands, all of these commercial species have the common drawback as laboratory animal: requirement of relatively large facility or tanks (especially for broodfish). They also require relatively long reproductive cycle than small aquarium fish such as zebrafish and medaka.

Our research group has been being trying to develop a novel transgenic model system using a new candidate fish species, mud loach (Misgurnus mizolepis). This small freshwater species has attractive merits from both small aquarium fish and commercially important fish species. Mud loach is important both for food and ceremonial works (by Buddhists) in Korea. Adult body size of mud loach is around 10 cm in total length, which is larger than zebrafish but much smaller than tilapia and carps. They can be easily maintained in laboratory aquarium throughout their whole life cycle. Mud loach embryo has soft chorion showing no adverse effect on microinjection, and the developing embryos are very transparent like zebrafish embryos, allowing the easy detection of wide range of reporter proteins based on immunohistochemistry and fluorescence analysis, which is often difficult in the species with opaque eggs like tilapia and salmonids (Nam et al., 1998, 1999). Mud loach is one of fish species with the fastest embryonic development. Approximately 24 hours after fertilization at 25 °C, mud loach embryos hatch, and the yolk sac absorption is generally completed within 48 hours post hatch (Nam et al., 2003). This time course is comparable to embryonic development of zebrafish and faster than that of most other fish species including tilapia (72–96 h, and embryos are nutritionally dependent on the yolk sac until approximately day 10). This species also represented short generation time with year round spawning under controlled condition. Average generation times are 3 months for male and 5 months for female, which is much shorter than tilapia, carp and salmonids, although relatively longer than zebrafish and
medaka. Mud loaches are very hardy with good tolerance to low oxygen (survivable at low level of dissolved oxygen near to zero since they are capable of intestinal respiration), temperature (survivable from near 0 °C to above 30 °C), and diseases. Furthermore this species produces a large number of eggs (more than 10,000 eggs per female, at one time) enough for performing any kinds of genetic experiments including transgenesis and chromosome-set manipulation. There is no need for collecting eggs from many broods to fulfill the number of eggs required, which is frequently unavoidable in many experiments with zebrafish and medaka. Finally, techniques for chromosome-set manipulations have already been well established in this species, including polyploidization (tri- and tetraploidy) and artificial parthenogenesis (gyno and androgenesis) (Kim et al., 1994; Nam et al., 2001c, 2004a).

One of the most important merits of fish system for vertebrate transgenesis is amenability for a wide range of chromosome-set manipulations (Streisinger et al., 1986; Powers, 1989). This attractive characteristic of the eggs allows us to perform a novel alternate approach for large-scale production of isogenic homozygous transgenic animals from a single heterozygous transgenic parent, using two forms artificial parthenogenesis: gynogenesis and androgenesis. Induced doubling of transgenic haploid genome originated from one sex without any contribution of genetic material from the other sex enables the production of isogenic transgenic animals of which all loci including the transgenic locus are completely homozygous. Production of isogenically stable homozygous transgenic lines can extend the utility of the transgenic animal in research by providing a minimized genetic background and facilitating stable transmission and expression of genetic and/or phenotypic markers. Furthermore, another round of induced parthenogenesis would make it possible to generate homozygous clonal transgenic population.

Somatic cell nuclear transfer approach that has been used for generation of cloned hemizygous transgenic animals (Schneike et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999) also requires the insertion of transgenes into the same site in multiple lines of animals for generating transgenic homozygous animals.

**Gene resources and wish list for transgenesis in fish**

Expressed sequence tag (EST) analysis, a powerful technique for rapid discovery of novel genes expressed have been performed to isolate candidate piscine genes for molecular genetic breeding of fish including transgenesis. In present, number of public entries in NCBI GenBank database (http://www.ncbi.nlm.nih.gov/dbEST) is over 28 millions, and fish entries correspond to about 3% of total ESTs. It can offer a fundamental basis for numerous genomic studies and other biological researches by providing powerful means to discover useful genes expressed in different tissues (Gong et al., 1994). This technique can also be of importance for illuminating a snapshot of a given genome (Douglas et al., 1999). Recently numerous EST projects have been developed in many finfish and shellfish species in order to (1) find useful piscine genes (Hamilton et al., 2000; Miyahara et al., 2000), (2) develop an efficient genetic marker for genome mapping (Liu et al., 1999) and (3) perform transcriptional profiling of different tissues under defined experimental stimuli (Kono et al., 2000; Savan and Sakai, 2002). Also the genetic information achieved in EST project will be much helpful to extend the “wish list” of transgenesis in fish (Nam and Kim, 2001).

Although transgenic manipulation in finfish has historical background of over 15 years, most transgenic applications to commercially important species have been targeted to the growth enhancement through transgenesis of GH gene. Only a few studies have performed on the other phenotypes such as temperature-resistance, disease resistance and modified metabolic pathway (Zbikowska, 2003). However,
further extensive examination on the performance of those transgenic fish should be made in future. To extend the “wish list” for transgenic application, numerous laboratories have performed the projects for gene discovery in bulk and have established the local databases regarding both sequence and expression of target genes (and also its transcripts). Major classes of genes receiving interests for theoretical and practical aspects in aquaculture are, of course, responsible for commercially important traits such as growth, viability, immune system, nutrition, stress-response and reproduction. These genetic resources will offer the opportunity and fundamental basis for initiating new projects of genomics and transgenesis in many fish species. Rapid extension of expression data of fish genes which is assisted by sophisticated modern platform tools (such as DNA microarray and proteome analysis) makes it possible to do transcriptional profiling of a given genome at a particular time. Based on the information on these expressed profiles, scientists are able to design various vector systems allowing the tissue-specific and/or targeted switching expression. The potential ‘wish list’ for current and on-going fish transgenesis except growth hormone transgenics is shown in Table 1 (See also Hackett and Alarez, 2000; Nam et al., 2002b).

### Table 1

<table>
<thead>
<tr>
<th>Target trait</th>
<th>Potential candidate genes for transgenesis</th>
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<tr>
<td>Enhanced resistance to diseases and stresses</td>
<td>Antimicrobial peptides (pleurocidin, hepcidin, cathelicidin etc)</td>
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<td>Lysozymes (C- and/or G-types)</td>
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<td>Ribozymes</td>
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<td>Antigenic protein (for genetic vaccines)</td>
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<td>Interferon</td>
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<td>Lectins</td>
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<td></td>
<td>Antioxidant enzymes</td>
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<tr>
<td>Vitamin C metabolism</td>
<td>Gulosonolactone oxidase</td>
</tr>
<tr>
<td>Digestive ability &amp; carbohydrate utilization</td>
<td>Cellulase</td>
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<td></td>
<td>Glucose transporter, hexokinase</td>
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<tr>
<td>Freeze resistance</td>
<td>Anti-freeze protein</td>
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<tr>
<td>Lipid metabolism</td>
<td>Apolipoproteins</td>
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<tr>
<td></td>
<td>Fatty acid desaturase</td>
</tr>
<tr>
<td>Sterility and reproductive control</td>
<td>Anti-sense GnRH</td>
</tr>
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<td></td>
<td>Aromatase</td>
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**Transgene delivery and analysis of transgenic fish**

Several transgene delivery methods have been used in many fish species but currently the most popular and reliable method is microinjection. This method simply involves the physical delivery of DNA
solution into embryo (mostly one-celled embryo just after insemination) using fine glass needle. Although fish eggs are abundant, relatively large and easily accessible, eggs from many fish species have characteristics that make microinjection difficult or troublesome. Fish eggs are surrounded by a chorion that rapidly hardens after fertilization, making it difficult to penetrate. Unlike mammalian eggs, fish eggs contained abundant yolk materials that make it extremely difficult to identify the position of the nucleus (chromosomes): it might be impossible to locate the injected DNA directly to the nucleus using fish eggs. For this reason, most microinjection trials in fish embryos were “blind injection”. In contrast to mice, the time from spermatozoa activation to the first cleavage in fish is quite short (for example, karyokinesis of the first cleavage in mud loach embryo is completed within 30 min at 25 °C) and early embryonic cell division is also rapid. It means there is only a short window of opportunity for microinjection into the early embryo, since theoretically (and ideally) the transgene should be injected at the single cell stage to expect the integration in most cells of transgenic organism. However, unfortunately even when the injection was made at one-celled embryos, late integration (integration of construct takes place after several rounds of cell cleavages) has commonly occurred in most gene transfer studies with fish, consequently leading the formation of mosaic founders.

To avoid the labor intensive procedures for microinjection and to overcome the limited time for gene transfer treatments, several candidate alternate strategies for transgene delivery to fish cells have been tried. Electroporation lets cell walls (membrane) temporarily much more porous by a short burst of electrical pulse and larger molecules like DNA can pass through. Owing to its simplicity of procedures allowing mass gene transfer to numerous fish embryos simultaneously, this technique has been encouraged as a useful alternate method for microinjection in early fish transgenic researches. However, a number of issues remain to be resolved especially regarding the extremely low viability of electroporation-treated embryos and low efficiency of genomic integration. As similar, several other mass gene transfer treatments have been employed. It include the (1) sperm-mediated transfer where spermatozoa are used as vectors delivering DNA to eggs based on the claims that DNA binds readily to the outer coat of spermatozoa, (2) biolistics in which microscopic particles (usually gold) are coated with DNA construct and explosively firing these particles directly to the cell through the cell membrane and (3) lipofection using synthetic lipid vesicles containing encapsulated DNA which can be taken up directly by animal cells. Researchers sometimes have tried gene transfer to fish cells by combining more than 2 methods (i.e. electroporation of lipofected sperm). Success rates greatly varied among fish species and working laboratories. Unfortunately there is currently no direct evidence that these mass gene transfer methods can offer a more effective or more efficient way to produce transgenic fish than microinjection: expression of foreign DNA introduced by those kinds of methods was successful during embryonic development in many cases, but mostly only transient without stable genomic integration.

Recently, several alternative methods based on the cell-mediated gene transfer have also been reported for making transgenic fish. Somatic nuclear transfer for animal cloning can be considered as one of alternative method for generating cloned transgenic fish lines. Cultured fish cells were manipulated in vitro, nucleus isolated from the recombinant cells, and the nucleus transferred to enucleated eggs from donor. Unlike mammalian system, nuclear transfer in fish doesn’t require the working process in utero. Similar strategies could also be made using embryonic stem cells (ESCs) technology (Hong et al., 2000). Another cell-mediated gene transfer method uses the primordial germ cells (PGCs) rather than somatic cells or embryonic cells (Takeuchi et al., 2002). All of those methods are considered as promising tool in
near future allowing the bridging *in vitro* and *in vivo* manipulations for fish transgenesis. Cell-mediated gene transfer in fish would be believed to overcome many drawbacks of conventional gene transfer methods caused by random integration, a high degree of mosaicism and an unpredictable expression pattern of the transgene.

**Manipulation of fish growth by transgenic technology**

Growth is one of the most important parameters affecting the success in farming practice of fish. Growth enhancement can provide advantages for aquaculture by shortening production times, enhancing feed conversion efficiency, and controlling product availability. For this reason, the growth has been the main target of transgenic engineering in commercially important fish species, and the most common type of engineering for this purpose has been carried out through the transfer of growth hormone (GH) gene constructs. In early phase of GH experiments in fish, phenotypic effects of GH transgene construct were only modest or negligible since transgene derived from very distantly related organisms would not work very well in host genome. In early experiments, availability of piscine GH genes was quite limited, and gene constructs used for fish transgenesis were generally comprised of mammalian GH genes (bovine, rat or human GH etc) driven by viral (CMV for example) or mammalian (mouse MT promoter for example) promoters (Zhang et al., 1990; Dunham et al., 1992). It is now widely accepted view that constructs homologous with respect to both regulatory and structural gene sequences appeared to be much more efficient than distantly heterologous constructs. The first clear evidence demonstrating the usefulness of all-piscine vector for growth enhancement was achieved in Atlantic salmon by use of an “all fish” chimeric growth hormone gene construct (Du et al., 1992). Thereafter extremely high growth rate effects have been obtained for some salmonid species including coho salmon using “all salmon” construct comprised of sockeye salmon GH fused to sockeye salmon MT promoter (Devlin et al., 1994; Pitkanen et al., 1999). Furthermore, dramatic growth acceleration (up to 35-fold) was observed in transgenic mud loach carrying an entire homologous transgene (Nam et al., 2001d). We constructed “all mud loach” transgene consisting of mud loach GH gene driven by mud loach beta-actin promoter, and transferred the transgene unit without vector sequence to mud loach embryo by microinjection. Consequently, mud loach containing this transgene unit in their genome should be called “autotransgenic” containing no heterospecific genetic material (Nam et al., 2001d). Autotransgenic strategy can be considered as a methodology to maximize the potential effectiveness of transgene using transgenic materials derived from the same species only. With similar fashion, the fast-growing transgenic carps (*Cyprinus carpio*) have also been developed by microinjecting carp GH expression vector into fertilized eggs. Transgenic carps also represented growth stimulation up to 30-fold at maximum and significantly improved feed conversion efficiency.

*Fig. 1. Six-month-old transgenic carps (3.1 and 3.3 kg) and non-transgenic siblings (140 and 160 g).*
Examples of significant growth enhancement in transgenic fish have also been achieved in several other farmed species including tilapias, salmons, carp and charr (Rahman et al., 1998; Krasnov et al., 1999; Mori and Devlin, 1999; Martinez et al., 2000). Generally, GH transgenic fish showed positive relationship between transgene expression and transgene copy integrated, although over expression of GH was always not accompanied by actual growth stimulation. Also the levels of enhanced growth were variable among transgenic lines: so far there is no clear direct evidence for a correlation between transgene expression and growth rate. Strain-specific growth rates suggest that characteristics of the insertion site and transgene structure play an important role in the expression and consequent physiological effect of the transgene. Poor correlations between transgene expression and growth may also be anticipated due to abnormalities arising from overexpression (Devlin et al., 1995; Nam et al., 2001d; 2002a). That is, maximal growth probably arises from moderate expression levels and slower growing fish arise from both lower and higher expression.

The phenotypic alterations observed in many GH transgenics are not limited to growth acceleration. Fast-growing transgenics revealed significantly improved feed conversion efficiency up to 1.9-fold (in transgenic mud loach), when compared to the non-transgenics. In these transgenics the GH may improve food and protein conversion by mobilization of lipids so that more of the ingested amino acids are available for protein growth (Weatherly and Gill, 1987; Rahman and Maclean, 1999). Generally, fast-growing transgenics are metabolically more efficient than non-transgenics: for example, the juvenile of transgenic tilapia showed higher protein synthesis and growth rate concomitant with enhanced glycolysis and increased oxidation of amino acids (Martinez et al., 2000). Increased metabolic rate and enhanced utilization of dietary lipids have also been reported in transgenic charr (Krasnov et al., 1999). In addition to growth enhancement, other phenotypic changes in fish morphology and some physiological abnormalities have been detected in some species: overexpression of GH in transgenic fish can result in pleiotropic effects, including skin color changes, modifications of skull shape, and acceleration of smoltification in salmonids, reduced fertility or even sterility due to a low level of sperm production, precocious sexual maturation and decreased viability. Effects on disease resistance, metabolism, endocrinology, swimming ability, organ structure, and behavior have also been observed in several species.

Finally, an interesting phenomenon observed in several GH transgenics is a gigantism beyond the normal adult size of those species. The most dramatic example is mud loach where autotransgenic loaches revealed extraordinary gigantic adult size that has never been recorded in this species. It is not clear at present whether or not this size (heavier than 400 g and longer than 40 cm) is exactly the biological maximum of this species. Similar gigantism has also been reported in GH-transgenic tilapia to grow above the normal size (4.2 kg at 2 years in transgenic, while the maximum sizes recorded in the wild is 3.8 kg) (Rahman et al., 1998). Further research should be made to study the detailed mechanism of gigantism beyond the normal size. Also the illustration of relationship between gigantism and lifetime of transgenics would be valuable to provide a good scope to address the regulation of growth and aging in vertebrates.

Genetic breeding, chromosome-set manipulation and safety issues of transgenic fish

Generation of stable isogenic transgenic lines, capable of transmitting the acquired novel traits
into their progenies at 100% frequency, would enhance the utility of transgenic fish by avoiding the laborious and time-consuming procedure for identifying and isolating transgenic offspring from nontransgenic siblings. Higher economic benefit through greater yield in group may also be expected by such homozygous fast-growing transgenic lines allowing 100% transgene frequency. Further, it may also facilitate stock management to avoid large size variations which occur when both fast growing transgenic and non-transgenic progenies are communally cultured. Homozygous transgenic lines can be generated by several ways: (1) sister–brother matings between heterozygous full-siblings (Kinoshita et al., 1996; Nam et al., 2000a), (2) by back crossing the heterozygous transgenics to their parental generation (Rahman et al., 1998), or (3) by selective induced parthenogenesis (see Thorgaard et al., 1992). When compared to the traditional methodologies, the induced parthenogenesis is more advantageous, as isogenesity and transgene homozygosity can be obtained in the transgenic line more or less simultaneously (Nam et al., 2000b). Isogenic homozygous lines have been established with fast-growing transgenic mud loach by artificial androgenesis. Homozygous androgenetic transgenic loaches displayed copy number dependent gene expression and showed the capacity to transmit the transgene to next generation at 100% rate (Nam et al., 2002a).

Fig. 2. Schematic diagram to show the establishment of isogenic homozygous transgenic fish by induced parthenogenesis from hemizygous transgenic fish.

Two successive rounds of artificial parthenogenesis from a hemizygous transgenic fish can generate the clonal homozygous transgenic population as exemplified by transgenic mud loach. Multi-locus DNA fingerprint profile of the progeny obtained by androgenic reproduction of isogenic homozygous transgenic fish showed that they were clonal relatives. Cloned transgenic homozygous offspring also showed an increased level of transgene expression with reduced variation among transgenic individuals when compared to those of non-cloned, hetero- and homozygous transgenics. This suggests
that a transgenic clonal fish population may be potentially useful with the viewpoint of minimized genetic background with relatively consistent transgene expression. Furthermore, the cloned fish in that manner were transgenic homozygous with transgene transmission to their subsequent generation at a 100% frequency. Those results suggest that combining technology of transgenesis and artificial parthenogenesis will rapidly make it possible to use the genetically pure isogenic homozygous transgenic system on a large scale in vertebrate transgenesis. This strategy for establishing isogenically stable homozygous transgenic lines can provide a potential way to overcome the major limitation of time requiring traditional sister–brother mating. Also the results demonstrate that the clonal transgenic homozygous fish line could simply be obtained in a large scale using this approach. The isogenic transgenic homozygous line (and the resultant clonal transgenic homozygous line) can extend the utility of transgenic animals in numerous research applications by providing a minimized genetic background and facilitating stable transmission and expression of marker transgenes. In addition, intraspecific hybridization between isogenic (or clonal) lines with different transgenic genotypes would be a good strategy, which may offer a new possibility to generate even better strains by combining desirable transgenic geno- and phenotypes from two different transgenic lines.

The commercial application of transgenic fish faces significant concern about the ecological risks, especially with regard to the reproductive interaction between transgenic fish and wild conspecifics (Dunham, 1999; Hallerman et al., 1999). To prevent ecological interactions between escaped farmed fish and wild members, both physical and reproductive containment measures have been proposed (Devlin and Donaldson, 1992). Sterilization by triploidy is believed as one of the most effective method for biological containment, however, the utility of triploidy as a containment method will depend on the efficacy of induction of the sterile condition on a large scale. Studies on triploid GH-transgenics have been made in several fish species including mud loach (Nam et al., 2001a), tilapia (Razak et al., 1999) and coho salmon (Devlin et al., 2004). Triploid GH transgenics in all of three species (loach, tilapia and salmon) have been found to grow slower than their diploid counterparts, albeit still much faster than their nontransgenic siblings. Although the reason for observed reduction in growth rate in triploids has not been clearly understood yet, the main reason responsible for this phenomenon might be the effective reduction of transgene dosage per cell and organism. Transgenes are present in one dose in three genomes in the triploids utilized in those experiments, compared to one dose in two genomes for diploid hemizygous transgenic fish. Because triploids have fewer larger cells than diploids (Benfey, 1999), the total number of GH transgenes per animal is also expected to be lower. One possible explanation is that triploid transgenics have reduced cell number than diploid transgenics, which may lead to lower amount of GH mRNA transcribed in many tissues, when compared to diploid transgenics, if the level of transgene expression per cell is the same between diploid and triploid. It is well known that increased DNA content resulting from triploidy leads to an increase in both nuclear and cellular volume in a wide range of tissues, but cell numbers are reduced to maintain normal organ and body size. Development of different transgenic triploid groups in a given transgenic line, in which transgenic groups contain different transgene copies per cell, will be valuable to examine possible relationship between transgene dosage and growth performance. Theoretically, if the recombination is not considered, the simple crossing hemizygous transgenic female with transgenic male followed by the second polar body retention will make it possible to generate different triploid genotypes with varying degree of transgene dosage per cell in a given transgenic line. Careful monitoring of the growth accelerations observed in these triploid
genotypes along with those of hemi- and homozygous diploid transgenic genotypes might offer useful system for addressing the effects of transgene copies and/or cell numbers on the regulation of transgene expression in a given transgenic line.

Although triploidy induction has been considered as one of methods for reproductive containment of transgenic fish, an important shortfall of triploidy is the occurrence of fertility in triploid fish (Pandian and Koteeswaran, 1998; Arai, 2001; Lee and Donaldson, 2001; Nam et al., 2001b). Many previous studies reported the possibility that triploid males might produce limited but potentially functional sperm (Kim et al., 1994; Van Eenennam et al., 1990), and, furthermore, in some cases, older triploids were found to be partly fertile even in females (Lee and Donaldson, 2001). Such limited but potential fertility of gametes from triploid fish may activate a portion of the gametes from wild conspecifics, resulting in a few viable offspring, and consequently cause unwanted gene contamination of local gene pool. It suggests that triploidization alone cannot always guarantee biological containment of transgenic fish. This problem of triploidy was partly resolved at least in transgenic mud loaches by induction of triploidy in the hybrid genotype. The transgenic triploid hybrids (allotriploids between cyprinid loach female and transgenic mud loach male) exhibited much more stringent sterility than transgenic autotriploid mud loach especially in males as evidenced by completely repressed gonads and no viable embryo (also absence of normal embryonic development) in fertilization trial using a suspension of minced testes from triploid hybrids (Nam et al., 2004b). Regarding the reproductive containment of transgenic fish, the generation of functional tetraploid breeding lines may be indispensable for the mass production of sterile triploid transgenic progenies by the simple, widely practicable method of interploid hybridization, (Chourrout et al., 1986; Guo et al., 1996). However the complete performance data on growth and reproduction of tetraploid fish are still quite limited unfortunately: relatively detailed information on the reproducibility of induced tetraploids was available only in rainbow trout and mud loach. Recent examination on the progeny test of tetraploid mud loach males (sperm DNA content and ploidy status of progenies) indicated the need for careful examination of the ploidy status of sperm from tetraploidy, whose somatic cells are distinctly characterized by 4n DNA content as the characterization of somatic cells with clear 4n DNA cannot always be used as an index for the production of diploid sperm. Therefore, confirmation on ploidy status of sperm of tetraploid males is a pre-requisite for the breeding program for generating triploid stock of genetically modified fish (Nam and Kim, 2004).

Several working groups in different countries are trying to prepare guidelines for the regulation of transgenic fish with respect to both food safety and ecological risks. It is no doubt we have to develop any regulations for transgenic fish based on global harmonization, and these regulations must address them in a way that fully considers the environmental impacts of their introduction into aquatic ecosystems. However, current regulatory approach to transgenic fish is unlikely to meet this standard yet. There ware too much uncertainties associated with prediction of ecological impacts of transgenic fish regarding both reproductive and non-reproductive interactions with wild members. Significant concerns regarding the use of transgenic fish in aquaculture must be mitigated to ensure that the fish are not a hazard to both human health and the environment. However, in many cases, the empirical data has not been generated to allow resolution of many of the issues. Long-term evaluations of transgenic fish are required to assess the stability of their phenotypic characteristics.
Perspective

Aquatic biotechnology can offer novel strategies to deal with the rising demands for fisheries products. Major platform technologies including recombinant DNA technology can make it possible that a new wave of research activities should emerge and new frontiers be created in fisheries and marine biosciences. The wealth of information provided by the numerous genomics projects in recent days including gene discovery, genome sequencing, and functional analysis of genetic materials has added new resources to the field of marine and aquatic biotechnology. Transgenic manipulation of fish will of course be assisted by such sophisticated modern technologies, and the progress towards productivity enhancement would be much more accelerated. Fish is considered as one of the best candidates for the first marketable transgenic animal but the biosafety of the transgenic fish in many aspects has to be carefully evaluated prior to entering the marketplace. Fish as bioreactors is most likely one of the promising directions in further development of gene expression system though transgenic manipulation.

Literature cited
