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Review

Application of Gene Transfer Technology for Genetic Improvement of Fish

Aliye SARMAŞIK
University of Çanakkale Onsekiz Mart, Aquaculture College, 17100, Çanakkale - TURKEY

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Abstract: The worldwide decline of natural fish stocks, the accumulation of chemical pollutants in aquatic environments and increasing world consumption of fish products has provided an impetus in many countries for a rapid growth in aquaculture. Fish produced from aquaculture currently account for over one-fourth of fish consumed by humans. However, success in aquaculture mainly depends on breeding, feeding and protection from disease. Application of recombinant DNA technology in aquaculture can help to improve these requirements.

Key Words: Transgenic fish, genetic improvement of fish, gene transfer, aquaculture

Balığın Genetik Islahı İçin Gen Transfer Teknolojisinin Uygulanması

Özet: Dünya çapında doğal balık stoklarında görülen azalma, doğal su ortamlarındaki kimyasal kirleticilerin artışı ve balık ürünlerinin tüketimi için artan talep pek çok ülkede kültür balıkçılığına karşı hızla artan bir yönelim sağlamıştır. Kültür balıkçılığı yoluyla üretilen balık miktarı hali hazırda insan tüketimine sunulan balık miktarının dörtte birini aşmış durumdadır. Ancak kültür balıkçılığındaki başarı esas olarak üretim, besleme ve hastalıklara karşı korunma gibi faktörlere dayanmaktadır. Rekombinant DNA teknolojisinin uygulanması bu faktörlerin iyileştirilmesine yardımcı olabilir.

Anahtar Sözcükler: Transgenik balık, genetik balık ıslahı, gen transferi, kültür balıkçılığı

Introduction

The demand for fish is, traditionally met from the natural population of fish in the world. However, the level of the total worldwide annual harvest of fish has plateaued at the maximal potential level of between 100 and 150 million metric tons per year (1). Moreover, the worldwide decline of the natural stocks of some commercially valuable fish species and the accumulation of chemical pollutants in aquatic environments have provided a strong impetus for aquaculture. Therefore, many countries have turned to aquaculture to increase fish production over the last several decades. The outcome of these efforts has been a yield of 30 millon metric tons per year, that clearly proves that aquaculture has great potential to significantly increase the world production of fish and to resolve the pressing problem of meeting world demand (2).

Success in the production of fish by aquaculture depends on several factors, such as a good genetic background of the broodstock, efficient prevention and detection of disease, understanding of the optimal physiological, environmental and nutritional conditions for growth and development, control of the reproductive cycle of the fish species, a supply of good quality water, and the application of innovative management skills (3). The newly developed technologies in molecular biology and biotechnology have provided the means, such as transgenic fish technology, to improve these factors. The application of transgenic fish technology can improve a number of factors, such as growth performance, disease resistance and adaptation to inappropriate environmental conditions. A number of investigators have already reported production of transgenic fish superior to their non-transgenic counterparts. For instance, Chen et al. demonstrated that tilapia expressing extra copies of trout growth hormon, gene grow much faster than their non-transgenic counterparts which do not posses extra copies of the growth hormone gene (4).

In the present paper, methods of gene transfer into fish and procedures used to characterize transgenic fish are summarized. In addition, the results of a gene transfer study performed to produce disease-resistant medaka (*Oryzias latipes*) (5) summarized as an example of the application of gene transfer technology for the production of genetically improved fish lines.

Principle of gene transfer

Transgenics are organisms into which heterologous DNA (transgene) has been artificially introduced and the transgene stably integrated into their genomes (6). Although transgenics have been produced since the early 1980s, the production of transgenic fish was accomplished only a decade ago, in the early 1990s (7). The main principle of gene transfer is to facilitate the transfer of heterologous DNA into the nucleus of a target cell where integration into the host genome takes place. This can be accomplished using a number of techniques, such as microinjection that allows delivery of the transgene directly into the nucleus, and electroporation that facilitates the formation of temporary pores on the surface of the target cells through which the transgene is introduced into the cytoplasm where it is then delivered to the nucleus by the cellular machinery (6).

Fish species used for gene transfer studies

To date, a range of fish species, such as Japanese medaka (*Oryzias latipes*), zebra fish (*Branchydanio rerio*), trout (*Onchorynchus mykiss*), Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis*), loach (*Misgurnus anguillicaudatus*) and goldfish (*Carassius aurata*), have been used for gene transfer studies. The species of fish to be used for gene transfer studies is determined according to the purpose of the study. Fish species that are small in size, such as Japanese medaka (*Oryzias latipes*), are chosen to test the efficiency of a method or to establish a model system for basic or applied studies (4). However, larger species such as tilapia are chosen if the transgenic fish will be used as a bioreactor or for aquaculture purposes (8).

Methods used for gene transfer

A variety of gene transfer techniques, such as calcium precipitation, microinjection, lipofection, retrovirus infection, electroporation, embryonic stem cells, sperm-mediated gene transfer and particle gun bombardment, have been used to produce transgenics. Different gene transfer techniques present various advantages and disadvantages depending on the species of animal to be used for gene transfer studies (6). For instance, methods such as microinjection and electroporation are currently used for gene transfer in egg-laying fish, whereas others, such as retroviral vector-mediated gene transfer, are used in live-bearing fish species (3). The methods currently used or under investigation for gene transfer in fish are summarized below.

Microinjection

Microinjection is considered the most effective method of gene transfer in high vertebrates such as mice and sheep. In this technique, the transgene is directly microinjected into the male pronuclei of fertilized eggs (6). However, the pronuclei of fertilized eggs in fish studied to date are not visible, and transgenes are usually injected into the egg cytoplasm. Although this method is successful in transferring DNA into fish embryos, it is a very tedious, laborious and time-consuming procedure for animals such as fish that produce a large number of eggs (3).

Electroporation

Electroporation utilizes a series of short electric pulses to permeate the cell membrane and allow the transgenes to enter the cells. The presence of a tough chorion layer around the fish eggs reduces efficiency, but removal of the chorion layer, physically or chemically, increases the efficiency of this technique. However, removal of the chorion is not applied routinely because it is a tedious procedure and introduces additional stress on newly fertilized eggs. Electroporation has been shown to be the most effective means of gene transfer in fish since a large number of fertilized eggs can be treated in a short time by this method (9,10). There are a number of commercially available electroporaters, such as Beakon 2000 (Baekon Co.), that can be effectively used for gene transfer studies in fish.

Sperm-mediated gene transfer

Sperm-mediated gene transfer has the potential to be one of the most favorable gene transfer techniques because of the nature of the procedure. It has been reported that in mice, spermatozoa are capable of binding DNA and carrying it into an egg. However, the sperm-mediated gene transfer procedure in mammalian systems is quite controversial at present, since the viability of sperm outside the reproductive tract is very short (a few minutes at most). On the other hand, fish spermatozoa can be stored in seminal plasma with little loss of viability for long periods (days). Therefore, this technique appears very promising for gene transfer in fish (3).

Lipofection

Lipofection enables encapsulation of DNA within a phospholipid bilayer and subsequent delivery into a cell. Liposomes have been used successfully as gene delivery systems in mammalian cell cultures (3). Although no attempts have been reported for gene transfer in fish using lipofection, this technique may also be applied for gene transfer in fish (11).

Retroviral infection

Retroviruses are ideal gene delivery vehicles due to their efficient integration and single copy insertion of genetic material into the host genome (3). Their succcessful use for gene delivery in higher vertebrates and in fish has been reported (12,13). However, the preparation of retroviral particles including the transgene of interest is a very laborious process, increases costs and requires technology. Therefore, use of this method is limited. Nevertheless this technique makes gene transfer possible for species such as live-bearers and crayfish, from which newly fertilized eggs cannot be readily obtained (14).

Among the techniques mentioned above, electroporation has been preferred in many laboratories for gene transfer in fish systems because of its efficiency, speed and simplicity.

Generation and Identification of Trangenic Fish

Transgene Constructs

A variety of genes that can be applied for the genetic improvement of aquatic animals have been isolated from host organisms and cloned by the use of recombinant DNA techniques. In general, previously cloned gene sequences are utilized as transgene constructs since cloning a new gene is a long and tedious procedure. A transgene construct usually contains a promoter sequence, a binding site for RNA polymerase to initiate transcription of the transgene message, a signal sequence to address where the transgene product is going to be expressed and the transgene sequence (Fig. 1).

Fig. 1. Structure of gene construct used to produce transgenic medaka resistant to bacterial infection. CMV: Cytomegalovirus promoter, Ig: catfish immunoglobulin signal peptide, cecropin: transgene sequence.

Egg Collection and Embryo Culture

Currently, the preferred way of producing a transgenic fish is to introduce transgene into a newly fertilized egg. For this purpose, fertilized eggs can be collected immediately after fertilization and maintained in ice-cold Yamamato's saline to slow down embryonic development for a short period of time before gene transfer (15).

Gene transfer

Gene transfer can be carried out using one of the techniques for generating transgenic fish mentioned above. For gene transfer in an egg-laying fish species such as medaka a Baekon 2000 (Baekon Co., CA) electroporation device can be used under the following conditions: pulse frequency, 2^6 pulse per cycle; burst time, 0.4 s; cycle number, 5; pulse width, 160 µS and distance of electrode from surface of buffer, 1 mm; voltage, 2.5-5.0 kv. Yamamoto's saline (16) can be used as the electroporation medium.

Gene transfer via retroviral vectors or lipofection can be accomplished by incubation of newly fertilized eggs or injection of gonads with retroviral vector or lipofection reagent packaged with the transgene of interest. In sperm-mediated gene transfer, the sperms are first incubated with transgene and then the eggs are fertilized with these treated sperms. After the gene transfer, treated embryos can be incubated under standard conditions established previously for each fish species until hatching.

Screening of Putative Transgenic Fish

It has been reported that only a part of gene tranfer trials are successful (3). Therefore, following the gene transfer, each individual needs to be analyzed for the presence of the transgene. For this purpose, following hatching, the treated individuals are reared to a stage at which a fin clip can be obtained without harming the individual to check for the presence of transgene. Genomic DNA from each surviving treated fish is prepared from a small piece of fin tissue fin clip. A variety of methods, such as dot blot and Southern blot hyridization, can be utilized to identify putative transgenic individuals using genomic DNA samples (15). However, Polymerase Chain Reaction (PCR) based assay and Southern blot analysis of the PCR products are currently commonly used in order to economically

handle a large number of samples. A transgene-specific oligomer set is used to amplify the transgene from the genomic DNA sample using a thermocycler. PCR products are then run on agarose gels and blotted to a nylon membrane. A radiolabeled transgene probe is used to identify the PCR products containing the transgene sequence blotted to the nylon membrane. Positive PCR products show the transgenic fish, and these are referred to as founder (P_1) individuals. P_1 individuals are then crossed with nontransgenic individuals to investigate the transmission of the transgene and to establish the transgenic fish lines. Progeny produced from the cross of a P_1 transgenic and nontransgenic individuals are referred to as the F_1 generation. F_1 transgenic individuals are identified by the same method used to identify P_1 transgenic individuals. A number of previous studies (3,4,9) demonstrated that some of the P_1 generation transgenic fish are germline mosaics, meaning that these individuals contain the transgene in only a part of their germ cells; these therefore, produce transgenic offspring in limited numbers (Fig. 2). F_1 transgenics are usually crossed with nontransgenic counterparts or with one another to obtain an F_2 generation and to determine whether there is a Mendelian inheritance of the transgene from F_1 progeny. F_2 generation individuals are identified by the same method used to identify former generations (Fig. 3).

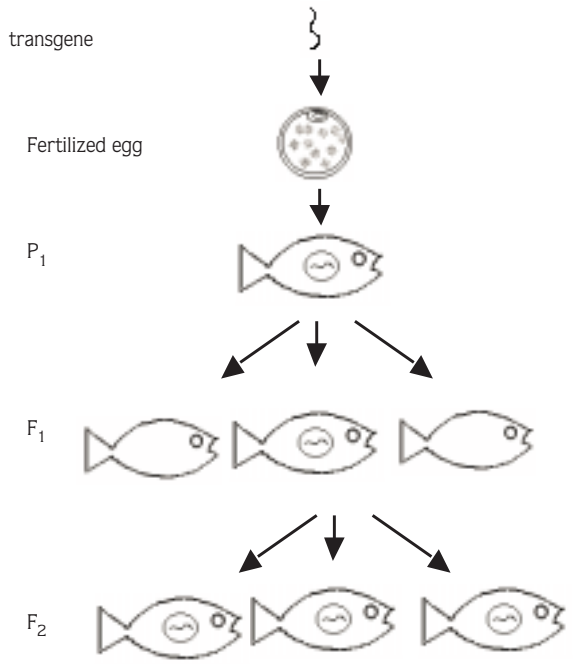

Fig. 2. Generation of transgenic fish lines. Transgene is transfered into newly fertilized eggs. Transgenic individuals (P_1) are determined among the adults hatched and reared from the treated eggs. The transgenic individuals among the P_1 progeny (F_1) produce transgenic individuals (F_2).

Expression and Inheritance of the Transgene in Transgenic Fish

If the transgene construct carries a functional promoter, a good number of transgenic individuals can be expected to express the transgene activity. The transgene expression can be inducible or constitutive depending on the structure of the promoter. Inducible promoters are preferred if constitutively active transgene expression is not favorable. Previous studies (3,6,9) showed that levels of transgene expression vary among transgenic individuals. This is because the numbers of integrated trasgenes and integration sites vary among different individuals. Zhang et al. (17) and Chen et al. (1) reported that among the P_1 and F_1 transgenic lines of common carp carrying rainbow trout growth hormone gene, the expression level of the transgene varied some tenfold.

Stable integration of the transgene is required for continuous transmission to subsequent generations to establish a transgenic fish line. To determine inheritance of the transgene, crosses are set up between transgenic individuals and nontransgenic control fish. Southern blot and hybridization of genomic DNA samples are commonly used to detect transgene integration. For this purpose, genomic DNA fragments are prepared by digesting genomic DNA from individuals of transgenic fish lines using restriction enzymes (RE). Following the RE digestion, genomic DNA samples are resolved on agarose gels and then transferred to nylon membranes. The nylon membranes are then hybridized with transgene specific probes radiolabeled as previously described elsewhere (15). After hybridization, nylon membranes are exposed to X-ray films at -80 ºC to vizualize the fragments harboring the transgene.

General Conclusion and Future Prospect

Classical genetic studies provided invaluble information about the genetic traits of organisms until the discovery of recombinant DNA technology. However, the discovery of recombinant DNA technology provided the impetus for scientists to utilize this technology to engineer a specific genetic trait in a directed fashion. The ability to introduce

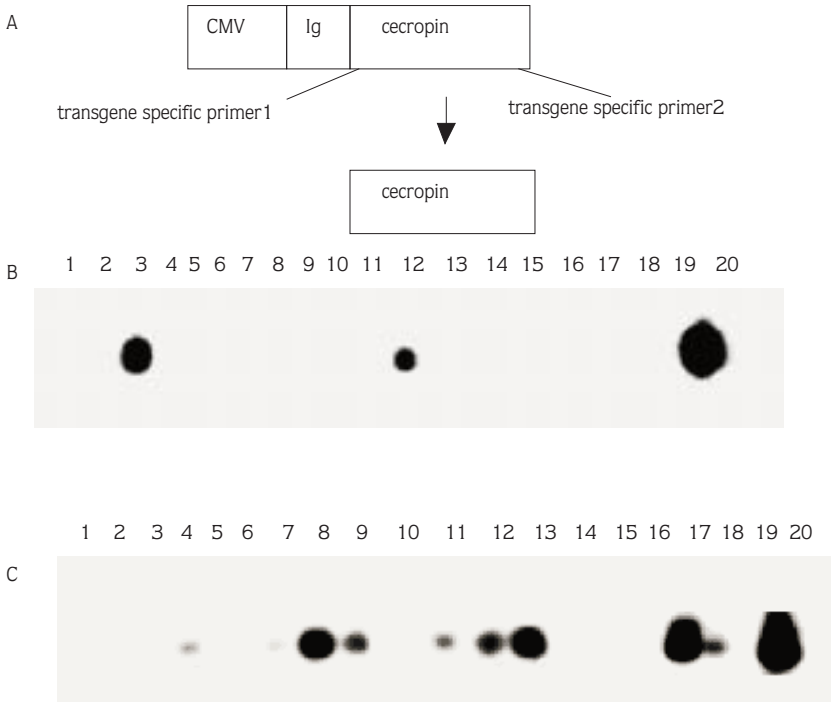

Fig.3. Identification of PCR positive transgenic individuals by PCR amplification of cecropin constructs from genomic DNA isolated from putative transgenic individuals. A: PCR amplification strategy of cecropin constructs with a transgene specific primer set. CMV: Cytomegalovirus promoter, Ig: catfish immunoglobulin signal peptide, cecropin:cecropin transgene sequence. B: Screening of P_1 generation. C: Screening of F_1 generation. 1: genomic DNA from nontransgenic control fish, 2-19: genomic DNA samples from putative transgenic fish, 20: positive control (plasmid DNA containing cecropin construct).

or knock out functional genes using this technology has provided a very powerful tool for scientists to understand biological processes and systems (6).

The first microinjection method enabling the introduction of a transgene into an organism was reported in 1966, and after a lapse of 15 years, the first transgenic mice were produced in the early 1980s. Since the production of the first transgenic mice, gene transfer technology has been pioneered using the mouse model, and the mouse continues to serve as a starting point for gene transfer procedures (6). However, fish systems have recently started to be accepted as models by many investigators in both applied and basic research, since fish systems possess several attractive attributes, such as genetic mechanisms that correspond to mice and human models, the ease of generating large numbers of animals in a short period compared to other vertebrate model organisms, and the ability to make genetic crosses among phenotypically diverse fish (4,18).

The complete sequencing of a human genome propelled science into a postgenome era. In this postgenome era, fish, as the most diverse vertebrate systems, represent a better candidate for promoting comparative genomics than any other system. It is clear that comparative genomic studies using different model organisms will facilitate the understanding of many important phenomena, such as physiological mechanisms. Transgenic fish generated by introducing or knocking out a functional gene of interest will be unique tools in unraveling the complex phenomena playing a major role in physiological processes. Therefore, new initiatives by a large number of investigators are currently focusing on the use of transgenic fish in biomedical research (18). For instance, researchers from a Canadian biotechnology company reported the use of transgenic tilapia carrying a human insulin gene for the transplantation of insulin-producing islets to humans. Since fish are further away from humans on the evolutionary scale, if transgenic fish are used as bioreactors, cross transfer of disease is much less likely (8).

Gene transfer into fish was accomplished in the early 1990s (13). However, since then many initiatives have been undertaken to achieve the goal of generating superior fish strains with improved growth rates, increased disease resistance, higher food conversion efficency, and many other desirable charcteristics. The research results reported in recent years from a number of laboratories and private companies have demonstrated that the production of transgenic fish strains with improved growth rates (4,19), cold resistance (20) and

increased disease resistance (5) can be achieved using this technology.

The applications of transgenic fish technology in aquaculture and biomedical research can provide a considerable number of benefits. However, it needs to be acknowledged that there are currently several major problems that need to be solved in order to utilize this technology efficently and safely. These problems include the absence of a convenient procedure for the mass identification of transgenic fish, random integration of a transgene that may potentially interrupt a functional gene in the host, and horizontal contamination or toxic effects (21) of the transgene on other organisms in the ecosystem. These problems can be solved by developing rapid and convenient procedures for the identification of transgenic fish, developing methods for targeting transgene integration sites in fish genome and assessing safety and the environmental impact of transgenic fish.

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