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The potential impact of modern biotechnology on fish aquaculture

Philippa Melamed [a,*], Zhiyuan Gong [a], Garth Fletcher [b], Choy L. Hew [a]

[a] *Department of Biological Sciences, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

[b] *Ocean Sciences Center, Memorial University of Newfoundland, St John's, Newfoundland, Canada*

Abstract

The introduction of molecular techniques in addition to the more traditional methods of biotechnology has supplied the resources to increase significantly production in world aquaculture. The ability to identify relevant genes endowing the phenotype of interest has certainly been helped by the ever-expanding databases, which have benefited not only from the various genome projects, but also from contemporary approaches such as the DNA chip, improved 2-D gel resolution and high throughput mass spectrometers. This, combined with improvements in transgenic technologies, has opened up vast possibilities to the aquacultural biotechnologist which include improving growth rates and cost-effectiveness, increasing resistance to pathogens and stressors, improving quality of broodstock and also creating the opportunity of making new or different products through altering their genetic make up. The platform technologies relevant to this field of functional genomics will be discussed in the context of applications beneficial to the field of aquaculture, while examples including those from our own research will be described. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The wealth of information provided by the various genome projects, and advances in functional genomics and transgenic technologies, have added new resources to the field of biotechnology. The potential application of these new approaches specifically to

* Corresponding author. Tel.: +65-8741882; fax: +65-8722013.
E-mail address: dbsmp@nus.edu.sg (P. Melamed).

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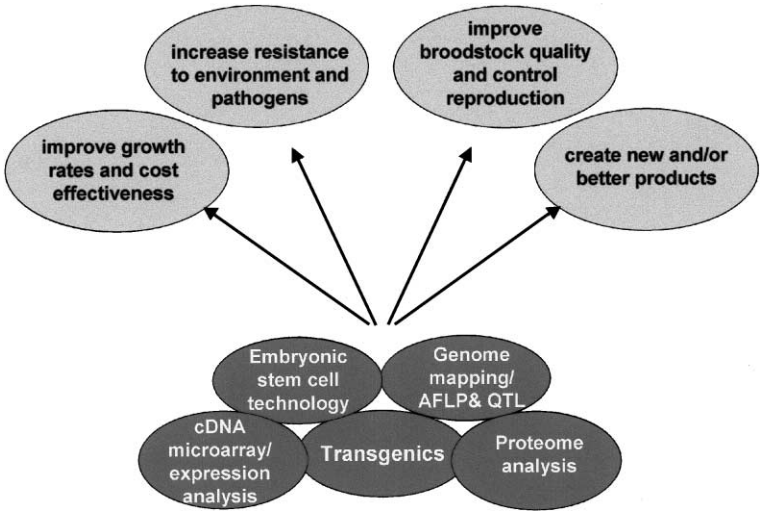

Fig. 1. An overview of some of the platform technologies (dark-shaded) in functional genomics, and their potential applications to aquaculture (light-shaded).

aquacultural biotechnology (summarized in Fig. 1) could bring us closer to achieving the growing demands of world aquaculture. These include the global needs of increased food production, discovery and development of new natural resources as well as awareness of the diminishing biodiversity and detrimental effects of modern society on the environment. In the following review some of the different areas of aquaculture which are being affected will be described. Examples, including those from our own research, will be given in an attempt to demonstrate the versatility of these new approaches.

2. An increase in food production

2.1. Growth enhancement

The ability to manipulate growth rates through the introduction of additional growth hormone (GH) genes was demonstrated originally in mice (Palmiter et al., 1982), but has been applied successfully to a number of other animals, including fish (e.g. Du et al., 1992; Devlin et al., 1994). Dramatic growth enhancement has been shown using this technique, especially in salmonids. In our work we have used an "all-fish" gene construct consisting of ocean pout antifreeze protein (AFP) promoter fused to Chinook salmon GH cDNA, injected into salmonid embryos. This particular promoter was used because it has been well characterized in our labs, showing a suitable pattern of tissue expression (mostly in the liver), and also lacking substantial seasonal variation in its activity. Moreover, the transcription factors required for its activation appear to be present in a large number of fish (e.g. medaka, Gong et al., 1991; salmon, Shears et al.,

1991; Devlin et al., 1995; goldfish, Wang et al., 1995; loach, Tsai et al., 1995), while the fact that it is not normally present in these other teleosts means that the transgene is easy to detect through simple PCR.

Such studies have revealed enhancement of growth in adult salmon to an average of 3–5 times the size of non-transgenic controls, with some individuals, especially during the first few months of growth, reaching as much as 10–30 times the size of the controls (Du et al., 1992; Devlin et al., 1994). Additional studies have utilized a number of other fish or non-fish promoters and similar although less dramatic effects have been seen than in salmonids. Some of these studies have also shown an increase in plasma GH levels, while the native pituitary GH seems to have been down-regulated as a result of increased negative feedback, resulting in smaller pituitaries and lower mRNA levels (Mori and Devlin, 1999). These fish generally appeared healthy, and some produced second and third generation transgenic offspring (Saunders et al., 1998). Moreover, the enhanced growth phenotype was inherited along with the genotype. The economic advantage of this kind of manipulation is obvious and in comparison with selective breeding methods, the time frame for reaching similar successes is likely to be most significant.

2.2. Control of reproductive activity

Traditional methods of spawning induction in fish have largely relied upon external stimulation of reproduction through simulation of stimulatory conditions, or direct administration of brain hormones (gonadotropin releasing hormone with, or without, dopamine antagonists), gonadotropins or pituitary homogenates. These methods are still commonly employed to induce spawning in aquaculture (e.g. Yaron, 1995; Peter and Yu, 1997). At the other end of the spectrum are those fish, such as tilapia, whose reproductive activity needs to be curbed because of small size at puberty. For these fish, monosex culture is preferred, which can be achieved by a number of methods. Most common is the treatment of fry with methyl testosterone to produce the masculine phenotype, although other methods such as specific crossing of closely related species with varying genetic make up (e.g. *Oreochromis niloticus* × *O. aureus*) can also result in hybrids of a single sex. For the production of sterile fish, with a high market value, triploidy can be used involving heat or pressure-shock to inactivate the sperm. Alternatively, gynogenetic individuals can be created and monosex culture then maintained through sex inversion (using steroids) to produce fertile males with the female genotype, enabling production of single-sex off-spring (Mair et al., 1997).

The introduction of transgenic techniques has put further emphasis on need for production of sterile progeny in order to minimize the risk of transgenic stocks mixing with the wild populations. However, these same technological developments have expanded the possibilities for producing either sterile fish or those whose reproductive activity can be specifically turned on or off using inducible promoters. The viability of this approach in vivo has been demonstrated in mice, in which specific genes, incorporating the Cre-loxP system, can be turned on through exposure to tetracycline or doxycycline (Kistner et al., 1996; Utomo et al., 1999). The adaptation of this system in fish would enable the stocking of fundamentally sterile fish which could be activated to

reproduce in culture as required. This would clearly be of considerable value, allowing both optimal growth and controlled reproduction of the transgenic stocks while ensuring that any escaped fish would be unable to breed.

The most promising method for repressing genes at present appears to be through use of antisense technology although in agriculture commercial success has only really been achieved so far in plants. The introduction of short DNA or RNA sequences corresponding to part of the coding sequence can be used to block expression of a particular protein either by binding the double stranded DNA helix to form a triplex at these particular bases, or by binding the mRNA, so blocking its processing and/or transportation. Alternatively, catalytic antisense sequences (ribozymes) act to cleave the target RNA at specific sites. Despite the promise heralded by antisense technology, there have been considerable problems in implementing this technique in animals, resulting primarily from difficulties in successful delivery to the target site and lethal side-effects due to non-specific actions (Gura, 1995). Thus, improvements or variations are now being sought to improve these techniques, including varying the structure of the antisense oligos to improve delivery (e.g. morpholinos: Summerton and Weller, 1997), or the use of double-stranded RNA (RNAi) which appears to silence gene expression specifically as long as the RNAi to be introduced is chosen carefully (Bosher and Labouesse, 2000).

The antisense approach is currently being pursued by a number of groups working on fish reproduction, and preliminary studies have shown that a construct of the promoter for salmon GnRH fused to the GnRH antisense cDNA, introduced into rainbow trout, leads to expression of the antisense RNA in the brain. This was accompanied by a decrease in the production of endogenous sGnRH mRNA in brain and pituitaries, although FSH and LH levels were not affected, and the fish reached sexual maturation (Uzbekova et al., 2000).

2.3. Increased resistance of fish to pathogens

The approaches taken to combat viral and bacterial pathogen damage to commercial stocks, are now turning to the use of DNA vaccines and antimicrobial agents. The former is based on injection of naked DNA encoding part of the antigen (usually a bacterial outer membrane or viral capsid protein) so that the protein will be expressed in vivo and the production of antibodies induced. This approach has already been used successfully in fish in a number of studies. An example is the injection of Atlantic salmon with a plasmid encoding infectious hematopoeitic necrovirus (IHNV) glycoprotein under the control of the cytomegalovirus promoter (pCMV): subsequent challenge with the virus 8 weeks later revealed that a significant degree of protection had been awarded. The same fish were still resistant 12 weeks later and it was shown that virus-neutralizing antibodies had been generated after the initial immunization, and that the titer increased after subsequent challenge (Traxler et al., 1999). A more recent study has shown that a single dose of nanogram quantities of the DNA is sufficient to elicit such a response (Corbeil et al., 2000). Similar protection was seen in rainbow trout after vaccination against viral haemorrhagic septicaemia virus (VHS), using the glycoprotein encoding sequence driven by pCMV (Lorenzen et al., 1999).

The availability of mass screening for antibodies with high affinity, through use of surface plasmon resonance (SPR), has enabled rapid advances in the field of producing recombinant antibodies. This technique examines the binding affinity between two molecules in real-time, based on the changes in the refractive index at the surface layer which are caused by the binding and are detected as changes in the SPR signal. Using this technology, a large number of antibodies can be simultaneously screened (e.g. through use of phage display libraries) for evaluation of the concentration of the molecules that recognize a broad-spectrum of pathogens.

The main disadvantage of these approaches is that they require quite detailed information on the structure and conformation of the pathogen's proteins, and even the optimal vaccine will still tend to have quite a narrow spectrum of effectiveness. Moreover, the logistical problem of administration of vaccines to large numbers of fish remains an obstacle that may be considerable.

Also in the context of providing immunity, antisense technology can be used and in fact the first reported case of using antisense oligonucleotides to block gene expression was for control of Rous sarcoma virus. In that example, sequences antisense to part of the 35S RNA of the virus were introduced to chick embryo fibroblast tissue cultures, resulting in inhibition of virus production (Zamecnik and Stephenson, 1978). However, here also, aside from the problems associated with antisense technology (as mentioned in the previous section), even if successful, this application will endow immunity only to the specific pathogen protein for which the antisense oligonucleotide was designed.

An alternative approach is to target the non-specific immune response through use of antimicrobial proteins, a large number of which are found in Eukaryotes. A recent review summarized some of these, and described two main groups based on chemical–structural characteristics (Andreu and Rivas, 1999). However, most of these have been poorly characterized, with the exception of the lysozyme which was shown already in 1985 to have a non-specific anti-bacterial effect (Austin and Allen-Austin, 1985). Currently our own research is directed at the production of transgenic fish carrying genes encoding a number of antimicrobial peptides, including lysozyme. As the levels of this protein are well correlated to the degree of disease resistance in salmonids (e.g. Demers and Bayne, 1997), it is expected that the transgenic fish harboring the ocean pout AFP promoter and trout lysozyme gene will demonstrate increased robustness in the challenge of a variety of microbes.

Immunostimulants, which facilitate the function of phagocytic cells and elevate their antibacterial activities, can also be used to increase resistance to disease, although the natural stimulation of these non-specific defence mechanisms is thought to be only temporary. Amongst the immunostimulants known to be effective in fish, glucan, chitin and levamisole enhance phagocytic activities, while yeast glucan and vitamin C also activate complement activity. In addition, levamisole and also growth hormone activate NK cells. A number of these have also been shown to enhance specific antibody responses (reviewed by Sakai, 1999).

The ability of these substances to increase resistance to environmental stress and their wide spectrum of activity make them particularly suitable for use in aquaculture, especially in comparison to, or even in complimenting the activity of vaccines. However, although immunostimulants can be administered by injection, careful dosage is

required, overdosing often leading to immunosuppression. Moreover, studies have shown the critical importance also of timing of the administration of these treatments, while clearly the injection itself (the preferred method of administration) may accentuate stress on the fish further while also being impractical in small fish. Thus, the way is open to develop genes which can be easily regulated, enabling activation of these immunostimulants at times when the fish are subject either to environmental stress (e.g. through use of promoters activated by high levels of cortisol), or to boost the immune reaction in the face of viral diseases (e.g. using interferon-responsive promoters).

2.4. Increased resistance of fish to cold temperatures

Cold-water temperatures pose a considerable stressor to many fish, and few are able to survive water temperatures much below 0–1 °C. Clearly this is often a major problem in aquaculture in temperate climates in which an unusually cold winter can annihilate entire stocks of sensitive fish. However, some marine teleosts have high levels (10–25 mg/ml) of serum antifreeze proteins (AFP) or glycoproteins (AFGP) which effectively reduce the freezing temperature by preventing ice-crystal growth. These proteins vary in structure, with one class of AGFP and four classes of AFP (Fletcher et al., 2001). Most of these are expressed primarily in the liver, and some are negatively controlled by growth hormone and show clear seasonal changes. In some fish, expression is also seen in the skin, gills and other peripheral tissues. The isolation, characterization and regulation of these antifreeze proteins, particularly of the winter flounder *Pleuronectes americanus*, has been the subject of our work for a considerable period, and we have examined the potential use of these proteins in lowering freezing temperatures in other species, particularly salmonids.

The gene encoding the liver AFP from winter flounder was successfully introduced into the genome of Atlantic salmon, where it became integrated into the germ line, and being passed on to the off-spring F3 where it was expressed specifically in the liver. Similar levels of the precursor proAFP (reaching a maximum of 200–400 μg/ml) were seen in all of the F3 transgenics and the serum was shown to have a characteristic hexagonal ice crystal pattern (as opposed to round ice-crystals in controls), indicating the presence of antifreeze activity. However, a number of Ala, Pro-specific endopeptidases which are required for production of the mature proteins, are lacking in Atlantic salmon, so antifreeze activity in these transgenic fish has not yet been optimized, reaching probably only 70% of its potential. Thus, the level of proAFP in the serum is below that of the winter flounder, so different techniques are still required to increase the copy number in order to elevate the expression of this gene to levels which would bestow freeze tolerance in the fish (Hew et al., 1999).

The introduction of AFPs to goldfish also increased their cold tolerance, to temperatures at which the control fish all died (12 h at 0 °C: Wang et al., 1995). Similarly, injection or oral administration of AFP to juvenile milkfish or tilapia led to an increase in resistance to a 26 to 13 °C drop in temperature. Most notably, only 3.4% of the AFP-treated tilapia died, as compared to 60% of the controls, while of the milkfish, 22.2% died after injection of a lower dose (100 μg/g bw), as compared to 70% of the controls (Wu et al., 1998). The development of stocks harboring this gene would clearly

be a major benefit in commercial aquaculture in countries where winter temperatures often border the physiological limits of these species.

Also in other vertebrates, this family of proteins has been used to help protect membranes from cold and freezing damage, possibly by altering their membrane structure (Rubinsky et al., 1992a,b). In this way, it appears that the membranes are bestowed with greater stability. The ability of these fish proteins to help in the preservation of sheep embryos at low (4 °C) temperatures has already been demonstrated, while also in cryopreservation AFP has been shown to protect pig oocyte oolemma from ice damage (Arav et al., 1993; Baguisi et al., 1997). The use of AFPs in cryopreservation of fish eggs and embryos still awaits further development.

2.5. Emerging new technologies for transgenics

Traditionally, transgenic fish have been produced largely through microinjection. However, the problem of mosaic expression of the transgenes is common, resulting in widely varying genotypes of the progeny. An example of experimentation with new and possibly more efficient ways for gene transfer is the use of pseudotyped pantropic retroviral vectors. These retroviruses are able to infect a particularly wide range of host cells and have been used to infect newly fertilized medaka eggs with a reporter gene, which appeared to become integrated into the entire germ line of some of the P1 females (Lu et al., 1997). Another study showed that pseudotyped retroviral infection and microinjection were equally efficient in passing the transgene onto zebrafish progeny, although there was wider variability in the extent of reporter gene expression among those founders that were microinjected (Linney et al., 1999).

The most promising tool for the future of transgenic fish production is undoubtedly in the development of the embryonic stem cell (ESC) technology. These cells are undifferentiated and remain totipotent, so they can be manipulated in vitro and subsequently reintroduced into early embryos where they can contribute to the germ line of the host. In this way, genes can be stably introduced or deleted. Despite the early success of ESC technology in mice, the technology has been slower to get going in fish, although early precursor cells have been cultivated from medaka (Mes 1 cells) and show many of the same features as mouse ESCs. Specifically, 90% of the host blastulae transplanted with Mes-1 cells developed into chimeric fry, and these cells became integrated into organs derived from all three germ layers, and differentiated into various types of functional cells (Hong et al., 1996, 1998, 2000).

3. Identification of desirable broodstock characteristics

3.1. Identifying genes involved in determining specific phenotypes:

The wealth of information coming from the research on fish genomes (e.g. zebrafish, medaka, and pufferfish) is providing new opportunities for identifying genes which are correlated to certain desirable traits. Clearly, however, even with the availability of this vast amount of data, much work must still be done to expound its meaning. The

approaches we are currently taking in this field of functional genomics include looking at differential gene expression through cDNA chip microarrays, and by comparing the entire proteomes of tissues or groups of cells subject to different treatments. In this way multiple genes can be identified, some of which will have useful applications in aquaculture.

The capability of the cDNA microchip to reflect changes in expression levels of tens of thousands of genes simultaneously is already having a huge impact on biological research. Its potential for application in drug design therapeutics in humans has been well recognized, providing both clues to potential drug targets and to their mechanisms of action (Lillie, 1997; Debouck and Goodfellow, 1999). Similar advances in aquacultural biotechnology have yet to be reported, although a number of groups are already using cDNA chips to evaluate changing mRNA levels in experimental animals, especially zebrafish where the number of EST (expressed sequence tag) clones is particularly extensive (e.g. Gong et al., 1997; to date 85,586 clones reported in NIH database). Recently, also from six different tissues of winter flounder 900 EST clones were generated, while around 300 EST clones are reported in the database for various tissues of the common carp, Nile tilapia and rainbow trout (Douglas et al., 1999; http://www.ncbi.nlm.nih.gov./dbEST/dbEST_summary.html). Extensive work is also being carried out on the Japanese flounder in an effort to find putative biodefence genes or those associated with the immune response: some 600 independent cDNA clones were identified from leukocytes infected with hirame rhabdovirus, and additional EST clones were isolated from spleen and liver (Inoue et al., 1997; Aoki et al., 1999; Nam et al., 2000). The growing availability of this kind of sequence information clearly will open up the possibilities for more extensive expression analysis on these species also.

Protein screening has become an excellent alternative approach of evaluating changes in expression levels accompanying physiological or pharmacological changes (see Table 1 and Fig. 2). The improvements in resolution of 2-D gels, combined with powerful

Table 1
A comparison of the cDNA microarray and proteomic approaches

cDNA microarray	Proteomics (2-D gel and MS)
mRNA levels may not predict accurately the levels of functional protein	Protein is the functional molecule: post-translational modifications can be detected
Expression of $>10{,}000$ genes can be measured simultaneously	Large chemical diversity can make separation difficult: especially limited for very basic or hydrophobic proteins
Difficult to achieve equal stringency across whole array, potentially lower specificity for longer probes	Some proteins may have solubility problems in concentrations required for detection
PCR can be used to amplify low abundant transcripts	Low abundant proteins will tend to get lost unless pre-selected
Cost involved in making a chip is considerable	Cost of running gels and analyzing proteins is relatively cheap

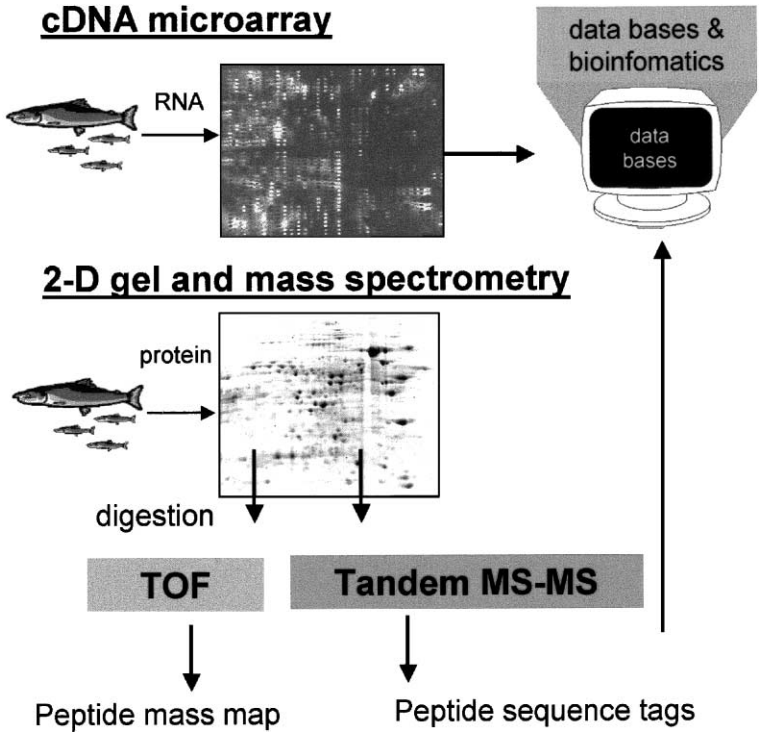

Fig. 2. A comparison of the cDNA microarray and proteomic approaches.

mass spectrometers such as the MALDI–time of flight (TOF) and tandem MS–MS, provide the precision of peptide mass maps and fragmentation patterns together with the capacity for high-throughput. Examples of some of the benefits of this technique have already been shown in mammals, in which proteins responding specifically to inflammation or toxins have been identified (Miller et al., 1999; Witzmann et al., 1999). An additional advantage of this approach over the cDNA microarray is that post-translational modifications can be identified. Clearly, these are particularly critical in cell signaling pathways, in which phosphorylation is often the means through which a protein is activated.

Some of our present studies using the proteomics approach include the search for antigenic proteins in white-spot shrimp virus (in order to develop suitable vaccines), detection of differentially regulated proteins in cell signaling of reproductive hormones (for manipulation of reproductive development and spawning) and the characterization of biologically active proteins in the incubation fluid surrounding the seahorse fry.

3.2. Molecular marker aided breeding programs

Breeding selection incorporating the use of molecular markers for specific traits is beneficial for enhancing characteristics which may be hereditary, such as growth rates, fecundity, disease resistance and features such as color and shape in ornamental fish.

The relevant genes can be mapped to genetic loci and fish selected for breeding programs using this marker.

The most commonly used genetic markers to date are the restriction fragment length polymorphisms (RFLPs) or microsatellites. For the first, restriction enzymes are used to cut DNA generating different length fragments due to the presence of absence of restriction sites and the numbers of insertions or deletions present in the intervening sequence of DNA. The addition of adaptors complimentary to the restriction sites used to cut the DNA, allows amplification of these fragments in an enhanced version of this technique, AFLP (amplified fragment length polymorphisms). The restricted fragments can then be probed using known sequences to produce a genetic map. An alternative approach is the use of microsatellites of tandem repeats which are duplicated many times (SSR: short sequence repeats) in a highly individual variation. Although each of these techniques has its own advantages and disadvantages, together they have contributed considerably to the tools available for genetic diversity research and genome mapping. In aquaculture, these methods have already been used to confirm the true gynogenetic identification of a number of species (e.g., Jenneckens et al., 1999; Felip et al., 2000; Zhou et al., 2000), and also to identify different stocks of the same species (e.g., Taggart et al., 1995; Mitchell et al., 1998; Verspoor et al., 1999).

A number of genomic maps have been created for the specific purpose of locating molecular markers for identifiable characteristics in fish. The first such map, for zebrafish, included 414 markers spaced at an average of 5.8 cM (Postlethwait et al., 1994). More recently, a map was produced with 2000 markers with an average resolution of 1.2 cM (Shimoda et al., 1999). A comprehensive genetic linkage map covering 1354.5 cM was also created for medaka, in which 633 markers were mapped and 24 linkage groups were detected, corresponding to the haploid number of chromosomes. Using this map, new genes can easily be mapped and DNA markers can be linked with specific phenotypes, while a number of genes for colour as well as those for several MHC-class I genes have already been mapped (Naruse et al., 2000).

Recently, this technique was used on rainbow trout that had been selectively bred for susceptibility or resistance to infectious hematopoeitic necrovirus (IHNV). RFLP using a locus containing the interferon-inducible genes, was able to discriminate seven distinct patterns, with one cross being identified that showed a correlation between homozygosity at this locus, and greater susceptibility to IHNV-caused mortality (Trobridge et al., 2000).

The quantitation of inherited traits can be performed using quantitative trait loci (QTL) which are identified by generating markers in an individual whose lineage is known, and creating a linkage map showing the relative distance between each of the markers. Statistical analysis is then carried out to find associations between these markers and the presence of the trait of interest. Clearly for this, a detailed linkage map with identification of many polymorphic loci dispersed throughout the genome is required. These can be constructed through use of AFLP markers, which has enabled QTL in a number of commercially grown plants. This approach is being employed in part of a molecular marker-assisted breeding program to produce new strains of tilapia from inter species crosses, with the aim of selecting for cold and salinity tolerance, and carcass quality through use of microsatellite and AFLP markers (Agresti et al., 2000).

4. Producing new products

4.1. Biosensors

Problems of poor water quality affect most developed countries in the world, the contaminants stemming largely from industrial waste and sewage. The effects of these pollutants have already been noted, particularly in fish, which often show reproductive dysfunction with males displaying feminization (Tyler et al., 1998; Sumpter, 1998). The biochemical responses of organisms to organic and metal compounds in the water can be measured and used as a biomarker for the level of pollution. Most commonly, cytochrome *P*4501A is used as it is responsive to a number of organic chemicals including aromatic hydrocarbons and dioxins. The induction of this gene by these contaminants is measured by changes in protein expression or mRNA levels. Alternatively, metallothioneins are utilized, which are induced specifically by metals (Livingstone, 1993).

At present these methods are used because mass screening is not available to detect the degree of contamination, largely because of the multiple ways in which the contaminants can operate (through direct binding to receptors, to promoter DNA or to co-activators and co-repressors). Thus, living biosensors remain valuable although this approach is limited because most of these biosensors are quite specific to certain groups of compounds and considerable work and expense are required for the assays. Alternative approaches are now being examined by our group, in which the gene encoding green florescent protein (GFP) is fused to a number of promoters which will respond to water pollutants. These include the promoters from some inducible genes such as: (i) those encoding heat shock proteins or metallothioneins which are induced by general stress, heavy metals or chemical toxins, (ii) those that contain estrogen response elements being induced by estrogens or xenoestrogens and (iii) tumor marker genes, which are induced by carcinogens (Gong, unpublished). The availability of the GFP as a reporter gene has enabled the use of transgenic organisms as continual qualitative biosensors for water contamination, providing rapid and visible results while eliminating the need for enzymatic or specific protein assays. Similar experiments have been carried out previously in yeast cells transformed with RAD54 fused to GFP; the cells turning green in response to DNA damage (Afanassiev et al., 2000).

4.2. New varieties of ornamental fish

The increasing world demand for ornamental fish has opened the market for new varieties with novel shapes or colors which can be supplied through the use of transgenics. Clearly this approach vastly increases the scope of possibilities and is more satisfactory than either the injection of dyes or selective breeding, which are the two methods currently used to widen the availability of phenotypes.

The availability of genes encoding additional fluorescent proteins, such as red (RFP), blue (BFP), yellow (YFP) and cyan (CFP), has enabled the production of green, red, blue, yellow or cyan fish in an almost endless variety of combinations. This innovation

has been taken up by one of our laboratories, in which novel colored fish are being successfully produced using the florescent color-encoding genes fused to a number of tissue-specific promoters. So far, GFP, RFP or YFP expression has been directed to the skin or skeletal muscles, allowing the green, red or yellow fish to be visualized under normal daylight. In addition, two-color fish were produced, showing green coloration in the skin, and red in the fast skeletal muscles (Ju et al., 1999). Such an approach, combined with selective breeding between fish carrying these different transgenes, could produce a wide array of multi-colored fish in future generations.

5. Concluding remarks

Since the first promises of transgenic animals were apparent when the introduction of additional growth hormone genes produced "gigantic" mice (Palmiter et al., 1982), optimism for the application of this and other transgenic technologies in all branches of agriculture has been widespread. In the ensuing years problems have been encountered because of the low efficiency of transformations, unexpected additional effects of the transgenes and also most recently hostility from the general public about the concept of genetic engineering, particularly regarding the food supply. At least in respect to fish, many of the technical problems have been overcome as shown in our own success in producing transgenic salmonids (Hew et al., 1995), although the test of the public's acceptance is still to come. The way ahead seems full of promise as the databases of genomic information increase and more efficient technologies will become available, thus presenting us with new opportunities to improve, increase and even create products in the field of fish aquaculture.

References

Afanassiev, V., Sefton, M., Anantachaiyong, T., Barker, G., Walmsley, R., Wolfl, S., 2000. Application of yeast cells transformed with GFP expression constructs containing the RAD54 or RNR2 promoter as a test for the genotoxic potential of chemical substances. Mutat. Res.-Genet. Toxicol. Environ. Mutat. 464, 297–308.

Agresti, J.J., Seki, S., Cnaani, A., Poompuang, S., Hallerman, E.M., Umiel, N., Hulata, G., Gall, G.A.E., May, B., 2000. Breeding new strains of tilapia: development of an artificial center of origin and linkage map based on AFLP and microsatellite loci. Aquaculture 185, 43–56.

Andreu, D., Rivas, L., 1999. Animal antimicrobial peptides: an overview. Biopolymers 47, 415–433.

Aoki, T., Nam, B.H., Hirono, I.I., Yamamoto, E., 1999. Sequences of 596 cDNA clones (565,977 bp) of Japanese flounder (*Paralichthys olivaceus*) leukocytes infected with *Hirame rhabdovirus*. Mar. Biotechnol. 1, 477–488.

Arav, A., Rubinsky, B., Fletcher, G., Seren, E., 1993. Cryogenic protection of oocytes with antifreeze proteins. Mol. Reprod. Dev. 36, 488–493.

Austin, B., Allen-Austin, D., 1985. Bacterial pathogens of fish. J. Appl. Bacteriol. 58, 483–506.

Baguisi, A., Arav, A., Crosby, T.F., Roche, J.F., Boland, M.P., 1997. Hypothermic storage of sheep embryos with antifreeze proteins: development in vitro and in vivo. Theriogenology 48, 1017–1024.

Bosher, J.M., Labouesse, M., 2000. RNA interference: genetic wand and genetic watchdog. Nat. Cell. Biol. 2, E31–E36.

Corbeil, S., LaPetra, S.E., Anderson, E.D., Kurath, G., 2000. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 8, 2817–2824.

Debouck, C., Goodfellow, P.N., 1999. DNA microarrays in drug discovery and development. Nat. Genet. Suppl. 21, 48–50.

Demers, N.E., Bayne, C.J., 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Dev. Comp. Immunol. 21, 363–373.

Devlin, R.H., Yesake, T.Y., Biagi, C.A., Donaldson, E.M., Swanson, P., Chan, W.-K., 1994. Extraordinary salmon growth. Nature 371, 209–210.

Devlin, R.H., Yesaki, T.Y., Donaldson, E.M., Hew, C.L., 1995. Transmission and phenotype effects of an antifreeze/GH gene construct in coho salmon (*Oncorhynchus kisutch*). Aquaculture 137, 161–169.

Douglas, S.E., Gallant, J.W., Bullerwell, C.E., Wolff, C., Munholland, J., Reith, M.E., 1999. Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes. Mar. Biotechnol. 1, 458–464.

Du, S.J., Gong, Z., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R., Hew, C.L., 1992. Growth enhancement in transgenic Atlantic salmon by the use of an "all-fish" chimeric growth hormone gene construct. Bio/Technology 10, 176–180.

Felip, A., Martinez-Rodriguez, G., Piferrer, F., Carrillo, M., Zanuy, S., 2000. AFLP analysis confirms exclusive maternal genomic contribution of meiogynogenetic sea bass (*Dicentrarchus labrax* L.). Mar. Biotechnol. 2, 301–306.

Fletcher, G.L., Hew, C.L., Davies, P.L., 2001. Antifreeze proteins of teleost fishes. Annu. Rev. Physiol. 63, 359–390.

Gong, Z., Vielkind, J.R., Hew, C.L., 1991. Functional analysis of promoter regions from fish antifreeze genes in transgenic Japanese medaka embryos. Mol. Mar. Biol. Biotechnol. 1, 64–72.

Gong, Z., Yan, T., Liao, J., Lee, S.E., He, J., Hew, C.L., 1997. Rapid identification and isolation of zebrafish cDNA clones. Gene 201, 87–98.

Gura, T., 1995. Antisense has growing pains. Science 270, 575–577.

Hew, C.L., Fletcher, G.L., Davies, P.L., 1995. Transgenic salmon: tailoring the genome for food production. J. Fish Biol. 47, 1–9.

Hew, C., Poon, R., Xiong, F., Gauthier, S., Shears, M., King, M., Davies, P., Fletcher, G., 1999. Liver-specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. Transgenic Res. 8, 405–415.

Hong, Y., Winkler, C., Schartl, M., 1996. Pluripotency and differentiation of embryonic stem cell lines from the medakafish (*Oryzias latipes*). Mech. Dev. 60, 33–44.

Hong, Y., Winkler, C., Schartl, M., 1998. Production of medakafish chimeras from a stable embryonic stem cell line. Proc. Natl. Acad. Sci. U. S. A. 95, 3679–3684.

Hong, Y., Chen, S., Schartl, M., 2000. Embryonic stem cells in fish: current status and perspectives. Fish Physiol. Biochem. 22, 165–170.

Inoue, S., Nam, B.H., Irono, I., Aoki, T., 1997. A survey of expressed genes in Japanese flounder (*Paralichthys olivaceus*) liver and spleen. Mol. Mar. Biol. Biotechnol. 6, 376–380.

Jenneckens, I., Muller-Belecke, A., Hörstgen-Schwark, G., Meyer, J.N., 1999. Proof of the successful development of Nile tilapia (*Oreochromis niloticus*) clones by DNA fingerprinting. Aquaculture 173, 377–388.

Ju, B., Xu, Y., He, J., Liao, J., Yan, T., Hew, C.L., Lam, T.J., Gong, Z., 1999. Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under zebrafish gene promoters. Dev. Genet. 25, 158–167.

Kistner, A., Gosse, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H., Bujard, H., 1996. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc. Natl. Acad. Sci. U. S. A. 93, 10933–10938.

Lillie, J., 1997. Probing the genome for new drugs and targets with DNA arrays. Drug Dev. Res. 41, 160–172.

Linney, E., Hardison, N.L., Lonze, B.E., Lyons, S., DiNapoli, L., 1999. Transgene expression in zebrafish: a comparison of retroviral and DNA microinjection approaches. Dev. Biol. 213, 207–216.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Tech. Biotechnol. 57, 195–211.

Lorenzen, N., Olesen, N.J., Koch, C., 1999. Immunity to VHS virus in rainbow trout. Aquaculture 172, 41–61.

Lu, J.K., Burns, J.C., Chen, T.T., 1997. Pantropic retroviral vector integration, expression, and germline transmission in mekada (*Ozyrias latipes*). Mol. Mar. Biol. Biotechnol. 6, 289–295.

Mair, G.C., Abucay, J.S., Skibinski, D.O.F., Abella, T.A., Beardmore, J.A., 1997. Genetic manipulation of sex ratio for the large-scale production of all-male tilapia, *Oreochromis niloticus*. Can. J. Fish. Aquat. Sci. 54, 396–404.

Miller, I., Haynes, P., Eberini, I., Gemeiner, M., Aebersold, R., Gianazza, E., 1999. Proteins of rat serum: III. Gender-related differences in protein concentration under baseline conditions and upon experimental inflammation as evaluated by two-dimensional electrophoresis. Electrophoresis 20, 836–845.

Mitchell, A., McCarthy, E., Verspoor, E., 1998. Discrimination of the North Atlantic lesser sandeels *Ammodytes marinus*, *A. tobianus*, *A. dubius* and *Gymnammodytes semisuamatus* by mitochondrial DNA restriction fragment patterns. Fish. Res. 36, 61–65.

Mori, T., Devlin, R.H., 1999. Transgene and host GH gene expression in pituitary and non-pituitary tissue of normal and GH transgenic salmon. Mol. Cell. Endocrinol. 149, 129–139.

Nam, B.H., Yamamoto, E., Hirono, I., Aoki, T., 2000. A survey of expressed genes in the leukocytes of Japanese flounder, *Paralichthys olivaceus*, infected with *Hirame rhabdovirus*. Dev. Comp. Immunol. 24, 13–24.

Naruse, K., Fukamachi, S., Mitani, H., Kondo, M., Matsuoka, T., Kondo, S., Hanamura, N., Morita, Y., Hasegawa, K., Nishigaki, R., Shimada, A., Wada, H., Kusakabe, T., Suzuki, N., Kinoshita, M., Kanamori, A., Terado, T., Kimura, H., Nonaka, M., Shima, A., 2000. A detailed linkage map of mekada, *Ozyrias latipes*: comparative genomics and genome evolution. Genetics 154, 1773–1784.

Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 30, 611–615.

Peter, R.E., Yu, K.L., 1997. Neuroendocrine regulation of ovulation in fishes: Basic and applied aspects. Rev. Fish Biol. Fish. 7, 173–197.

Postlethwait, J.H., Johnson, S.L., Midson, C.N., Talbot, W.S., Gates, M., Ballinger, E.W., Africa, D., Andrews, R., Carl, T., Eisen, J.S., Horne, S., Kimmel, C.B., Hutchinson, M., Johnson, M., Rodriguez, A., 1994. A genetic-linkage map for the zebrafish. Science 264, 699–703.

Rubinsky, B., Mattioli, M., Arav, A., Barboni, B., Fletcher, G.L., 1992a. Inhibition of $Ca2+$ and $K+$ currents by antifreeze proteins. Am. J. Physiol. 262, R542–R545.

Rubinsky, B., Arav, A., DeVries, A.L., 1992b. The cryoprotective effect of antifreeze glycopeptides from Antarctic fishes. Cryobiology 29, 69–79.

Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture 172, 63–92.

Saunders, R.L., Fletcher, G.L., Hew, C.L., 1998. Smolt development in growth hormone transgenic Atlantic salmon. Aquaculture 168, 177–193.

Shears, M.A., Fletcher, G.L., Hew, C.L., Gauthier, S., Davies, P.L., 1991. Transfer, expression and stable inheritance of antifreeze protein genes in Atlantic salmon, *Salmo salar*. Mol. Mar. Biol. Biotechnol. 1, 58–63.

Shimoda, N., Knapik, E.W., Ziniti, J., Sim, C., Yamada, E., Kaplan, S., Jackson, D., de Sauvage, F., Jacob, H., Fishman, M.C., 1999. Zebrafish genetic map with 2000 microsatellite markers. Genomics 58, 219–232.

Summerton, J., Weller, D., 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucl. A 7, 187–195.

Sumpter, J.P., 1998. Xenoendocrine disrupters—environmental impacts. Toxicol. Lett. 103, 337–342.

Taggart, J.B., Verspoor, E., Galvin, P.T., Moran, P., Fergusuon, A., 1995. A minisatellite DNA marker for discriminating between European and North American Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 52, 2305–2311.

Traxler, G.S., Anderson, E., LaPetra, S.E., Richard, J., Shewmaker, B., Kurath, G., 1999. Naked DNA vaccination of Atlantic salmon, *Salar salar* against IHNV. Dis. Aquat. Org. 38, 183–190.

Trobridge, G.D., LaPetra, S.E., Kim, C.H., Leong, J.C., 2000. Mx mRNA expression and RFLP analysis of rainbow trout *Oncorhynchus mykiss* genetic crosses selected for susceptibility or resistance to IHNV. Dis. Aquat. Org. 40, 1–7.

Tsai, H.J., Tseng, F.S., Liao, I.C., 1995. Transgenic loach: electroporation of sperm to introduce foriegn DNA. Can. J. Aquat. Sci. 52, 776–787.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28, 319–361.

Utomo, A.R.H., Nikitin, A.Y., Lee, W.-H., 1999. Temporal, spatial, and cell-type specific control of Cre-mediated DNA recombination in transgenic mice. Nat. Biotechnol. 17, 1091–1096.

Uzbekova, S., Chyb, J., Ferriere, F., Bailhache, T., Prunet, P., Alestrom, P., Breton, B., 2000. Transgenic rainbow trout expressed sGnRH-antisense RNA under the control of sGnRH promoter of Atlantic salmon. J. Mol. Endocrinol. 25, 337–350.

Verspoor, E., McCarthy, E.M., Knox, D., 1999. The phylogeography of European Atlantic salmon (*Salmon salar* L.) based on RFLP analysis of the ND1/16sRNA region of the mtDNA. Biol. J. Linn. Soc. 68, 129–146.

Wang, R., Zhang, P., Gong, Z., Hew, C.L., 1995. Expression of the antifreeze protein gene in transgenic goldfish (*Carassius auratus*) and its implication in cold adaptation. Mol. Mar. Biol. Biotechnol. 4, 20–26.

Witzmann, F.A., Fultz, C.D., Grant, R.A., Wright, L.S., Kornguth, S.E., Siegel, F.L., 1999. Regional protein alterations in rat kidneys induced by lead exposure. Electrophoresis 20, 943–951.

Wu, S.-M., Hwang, P.-P., Hew, C.L., Wu, J.-L., 1998. Effects of antifreeze protein on cold tolerance in juvenile tilapia (*Oreochromis mossambicus* Peters) and milkfish (*Channos channos* Forskaal). Zool. Sci. 37, 39–44.

Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129, 49–73.

Zamecnik, P.C., Stephenson, M.L., 1978. Inhibition of *Rous sarcoma* virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. U. S. A. 75, 280–284.

Zhou, L., Wang, Y., Gui, J.F., 2000. Analysis of genetic heterogeneity among five gynogenetic clones of silver crucian carp, *Carassius auratus gibelio* Bloch, based on detection of RAPD molecular markers. Cytogenet. Cell. Genet. 88, 133–139.