DEVELOPMENT OF LIVING COLOR TRANSGENIC MEDAKA LINES FOR BIOMONITORING AQUATIC CONTAMINATION

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Ng Hwee Boon Grace

12th April 2013

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Table of Contents

Declaration **i**

Acknowledgments **ii**

Table of Contents **iii**

Summary **viii**

List of Tables **x**

List of Figures **xi**

List of Common Abbreviations **xiv**

1. Introduction **1**

1.1 Water pollution and water quality evaluation 2

1.2 Biomarkers in toxicology 5

1.3 The use of transgenic fish as water sentinel 6

1.3.1 Advantages of transgenic fish model system 6

1.3.2 Small fish models as biomonitoring sentinels 8

1.3.3 Examples of biomonitoring transgenic fish 9

1.4 Transcriptional response to environmental chemicals 13

1.4.1 Pollutant response elements 13

1.4.2 Heat shock protein 70 15

1.4.2.1 The role of HSP70 as biomarker 15

1.4.2.2 HSP70 signaling pathway 17

1.4.3 Cytochrome P450 1a1 18

1.4.3.1 The role of CYP1a as biomarker 18

1.4.3.2 CYP1a signaling pathway 21

1.5 Aims and objectives in this study 21

2. Materials and Methods **26**

2.1 Medaka fish care and generation of transgenic medaka 27

2.1.1 Fish Husbandry 27

2.1.2 Spawning 27

2.1.3 Microinjection 27

2.1.4 Screening for transgenic founders 28

2.2 Molecular techniques 29

2.2.1 Genomic DNA extraction 29
2.2.2 RNA extraction 30
2.2.3 Polymerase chain reaction 31
2.2.4 One step reverse transcription PCR 33
2.2.5 Synthesis of *Ac* mRNA for microinjection 33
2.2.6 Whole mount *in situ* hybridization 34
2.2.6.1 Synthesis of DIG labeled RNA probe for *in situ* hybridizations 34
2.2.6.2 Fixation and proteinase K digestion of the embryos 34
2.2.6.3 Hybridization of DIG probes 35
2.2.6.4 Antibody incubation and staining 36
2.3 Plasmid constructs 37
2.3.1 Restriction endonuclease digestion of DNA 37
2.3.2 Ligation 37
2.3.3 Transformation and retransformation 37
2.3.4 Colony screening 38
2.3.5 Plasmid amplification and purification 39
2.3.6 DNA sequencing 39
2.3.7 DNA vectors 40
2.3.7.1 pDs(KRT4-EGFP) 40
2.3.7.2 pDs(HSP70-EGFP) 40
2.3.7.3 pDs(CYP1a-EGFP) 41
2.4 Analysis of genomic insertions 41
2.4.1 Southern blot analysis 41
2.4.1.1 Synthesis of DIG-labeled DNA probe 41
2.4.1.2 DNA digestion and separation 42
2.4.1.3 Southern blot transfer 42
2.4.1.4 Southern blot hybridization 43
2.4.1.5 Antibody incubation of Southern blot 43
2.4.1.6 Stripping of probe 44
2.4.2 Linker-mediated PCR (LM-PCR) 44
2.4.3 Basic Local Alignment Search Tools (BLAST) analysis 47
2.5 Treatments of the transgenic embryos and adult 47
2.5.1 Chemicals stock solution preparation 47
2.5.2 Chemical exposure treatment 48
2.5.2.1 Chemical exposure treatment of larvae 48

2.5.2.2 Chemical exposure treatment of adult fish 48
2.5.3 Heat shock treatment 49
2.5.4 Fluorescence and image capture 49

3. Result 50
3.1 Characterization of two inducible medaka promoter 51
3.1.1 Inducible expression of *hsp70* and *cyp1a* mRNAs in medaka embryos 51
3.1.1.1 Up-regulation of *hsp70* by heat shock and mercury treatment 51
3.1.1.2 Up-regulation of *cyp1a* by TCDD and BAP treatments 52
3.1.2 Analysis of response elements in promoter region 55
3.1.2.1 *hsp70* promoter and hsp70-EGFP construct 55
3.1.2.2 *cyp1a* promoter and cyp1a-EGFP construct 57
3.1.3 Transient transgenic analyses 59
3.1.3.1 Induction of GFP expression of pDs(HSP70-EGFP)-injected embryos by heat shock and mercury 59
3.1.3.2 Induction of GFP expression in pDs(CYP1a-EGFP)-injected embryos by BAP and TCDD treatment 61
3.2 Using the maize Ac/Ds transposon to develop transgenic medaka 63
3.2.1 Enhanced transient transgenic expression using Ac/Ds maize transposon in medaka 63
3.2.2 Generation of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) transgenic medaka 67
3.2.3 Variable constitutive and induction GFP expression in F1 generation 69
3.2.4 Multiple insertions in founder’s family 74
3.3 Selection of F1 for establishing biomonitoring lines 77
3.3.1 Determination of transgene insertion number by Mendelian inheritance 77
3.3.2 Confirmations of single insertion by Southern blot hybridization 79
3.3.3 Analysis of flanking sequences 81
3.3.4 Description and selection of best transgenic lines for biomonitoring purpose 88
3.3.4.1 Tg(*hsp70:gfp*) line 88
3.3.4.2 Tg(*cyp1a:gfp*) lines 93
3.4 Characterization of Tg(*hsp70:gfp*) for biomonitoring pupose 100

3.4.1 Heavy metals exposure of Tg(*hsp70:gfp*) fry 100
3.4.2 Exposure of Tg(*hsp70:gfp*) fry to other pollutants 105
3.4.3 Mercury exposure of adult Tg(*hsp70:gfp*) fish 112
3.4.4 Conclusion of Tg(*hsp70:gfp*) as a biomonitoring fish 116
3.5 Characterization of Tg(*cyp1a:gfp*) for biomonitoring application 117
3.5.1 PAH exposure of Tg(*cyp1a:gfp*) 117
3.5.2 Exposure of Tg(*cyp1a:gfp*) fry to other pollutants 123
3.5.3 TCDD exposure of adult Tg(*cyp1a:gfp*) fish 128
3.5.4 Conclusion of Tg(*cyp1a:gfp*) as biomonitoring fish 130

4. Discussion 131
4.1 Selection of inducible promoters 132
4.1.1*hsp70* promoter 132
4.1.2 *cyp1a* promoter 134
4.2 Use of maize Ac/Ds transposon system to generate transgenic medaka 136
4.2.1 Efficient germ-line transmission of transgene in medaka by using Ac/Ds transposon 137
4.2.2 Typical transition of Ac/Ds is retained in transgenic medaka 138
4.2.3 Genomic analysis of integration sites 140
4.2.4 The potential of Ac/Ds transposon system in gene/enhancer trap in medaka 141
4.3 Chemical exposure of Tg(*hsp70:gfp*) 143
4.3.1 Differential induction of GFP expression in different tissues by different heavy metals 143
4.3.2 Transgenic GFP expression in mercury treatment 144
4.3.3 Transgenic GFP expression in cadmium treatment 145
4.3.4 Transgenic GFP expression in arsenic treatment 146
4.3.5 Other chemical treatments 147
4.3.6 GFP induction in adult Tg(*hsp70:gfp*) medaka 148
4.3.7 Conclusion on Tg(*hsp70:gfp*) 149
4.4 Chemical exposure of Tg(*cyp1a:gfp*) 149
4.4.1 GFP expression is observed in similar organs in both PAHs and dioxin treatments 149
4.4.2 Transgenic GFP expression in 3-MC treatment 151
4.4.3 Transgenic GFP expression in BAP treatment 151
4.4.4 Transgenic GFP expression in TCDD treatment 152

4.4.5 Other categories of pollutants 154
4.4.6 GFP induction in adult Tg(*cyp1a:gfp*) medaka 154
4.4.7 Conclusion on Tg(*cyp1a:gfp*) 155
4.5 Summary 155
4.5.1 Limitations and improvements 156
4.5.2 Future directions 158

References **160**

Summary

With the advent of Green Fluorescent Protein (GFP) reporter gene, it is feasible to apply the living color transgenic fish for monitoring of water contamination. Here, we report the generation of two biomonitoring transgenic medaka lines using a stress-inducible promoter, *hsp70*, and xenobiotic inducible promoter, *cyp1a,* to express the GFP reporter gene amd these two transgenic lines are designated as Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) respectively. A fairly novel transposon tool in fish transgenesis, maize Ac/Ds, was used to aid the generation of these transgenic medaka. High germline transmission rates and classic features of Ac/Ds transposon system were observed, hence demonstrating the high efficiency of Ac/Ds to aid transgene integration in medaka. Our lab has identified five different categories of chemical pollution in water bodies and both Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) medaka were exposed to at least one chemical from each category to determine their inducibility as well as their sensitivity. For Tg(*hsp70:gfp*), GFP expression was detected in heavy metal exposure such as mercury, arsenic and cadmium but not significantly detected in other category of pollutantss such as 4-nitrophenol, bisphenol A *etc.* . Interestingly, specific heavy metals invoke specific GFP expression patterns in the embryos. Thus the newly developed transgenic line may be useful for monitoring environmental stresses caused by heavy metals and possibly establish a pattern database to identify various types of heavy metal insults. As for Tg(*cyp1a:gfp*), strong GFP expression was detected in organs including liver, kidney and gut when treated with polycyclic aromatic hydrocarbon (PAH) and dioxin such as 3-methylcholanthrene, benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzodioxin. However, GFP expression was insignificant in other chemical exposures such as 4-nitrophenol, bisphenol A etc. of Tg(*cyp1a:gfp*). These observations indicate the

potential use of Tg(cyp1a:gfp) for monitoring polyaromatic hydrocarbon(PAH) contaminations.

List of Tables

Table 1.1	Examples of biomonitoring transgenic fish	12
Table 1.2	Summary of response element to environmental pollutants	14
Table 1.3	List of some chemicals that induced expression of *hsp70* in aquatic organisms.	16
Table 1.4	List of some chemicals that induced expression of *cyp1a* in aquatic organisms.	20
Table 1.5	List of toxicants used in the study.	25
Table 2.1	List of primers used for various PCR reactions.	32
Table 2.2	List of primers used in LM-PCR.	46
Table 3.1	Frequency of transgenic progeny of positive pDs(HSP70-EGFP) and pDs(CYP1a-EGFP) founders.	68
Table 3.2	Transgene inheritance ratio of selected transgenic F1 individual.	78
Table 3.3	Observations of preliminary 72 hrs exposure of chemicals from various classes of pollutants using 1-3 dph wild type fry.	106
Table 3.4	Summary of GFP induction of Tg(*hsp70:gfp*) fry by various chemical exposure.	116
Table 3.5	Summary of GFP induction in Tg(*cyp1a:gfp*) fry by various chemical exposure.	130

List of Figures

Figure 1.1	Different chemical classes of environmental pollutants.	24
Figure 3.1	Induction of *hsp70* mRNA by heat shock and mercury.	53
Figure 3.2	Induction of *cyp1a* mRNA by TCDD and BAP.	54
Figure 3.3	Schematic diagram of *hsp70* promoter and *hsp70*-GFP construct, pDs(HSP70-EGFP).	56
Figure 3.4	Schematic diagram of *cyp1a* promoter and *cyp1a*-GFP construct, pDs(CYP1a-EGFP).	58
Figure 3.5	Transient GFP expression of pDs(HSP70-EGFP)-injected 2-dpf embryos under heat shock.	60
Figure 3.6	Transient GFP expression of pDs(HSP70-EGFP)-injected 3-dpf embryos induced by mercury chloride.	60
Figure 3.7	Transient GFP expression of pDs(CYP1a-EGFP)-injected 3-dpf embryos induced by BAP.	62
Figure 3.8	Transient GFP expression of pDs(CYP1a-EGFP)-injected 3-dpf embryos induced by TCDD.	62
Figure 3.9	Analysis of transient GFP reporter expression using Ac/Ds transposons system with pDs(KRT4-EGFP).	65
Figure 3.10	Analysis of transient GFP reporter expression using Ac/Ds transposons system with pDs(HSP70-EGFP).	66
Figure 3.11	Heat shock inducible GFP expression in 6-dpf F1 embryos of Tg(*hsp70:gfp*).	71
Figure 3.12	GFP expression in F1 embryos of Tg(*cyp1a:gfp*).	72-73
Figure 3.13	Analysis of transgene insertion.	76
Figure 3.14	Demonstration of single insertion in selected transgenic lines by Southern blot hybridization.	80
Figure 3.15	Flanking sequence of transgene integration.	83
Figure 3.16	Identification of transgenic insertion site in Tg(*hsp70:gfp*) 1.1.	84
Figure 3.17	Identification of transgenic insertion site in Tg(*cyp1a:gfp*) 1.1.	85
Figure 3.18	Identification of transgenic insertion site in Tg(*cyp1a:gfp*) 3.2.	86

Figure 3.19 Identification of transgenic insertion site in Tg(*cyp1a:gfp*) 4.2. 87

Figure 3.20 Constitutive GFP expression of Tg(*hsp70:gfp*) 1.1. 90

Figure 3.21 Heat shock induced GFP expression in Tg(*hsp70:gfp*) 1.1 F1 embryos. 91

Figure 3.22 Dosage-dependent induction of GFP expression by $HgCl_2$ in F1 embryos of Tg(*hsp70:gfp*) 1.1. 92

Figure 3.23 Constitutive GFP expression of Tg(*cyp1a:gfp*) 3.2. 96

Figure 3.24 TCDD induction of GFP expression in selected Tg(*cyp1a:gfp*) lines. 97

Figure 3.25 Corresponding endogenous expression of *cyp1a* gene to GFP expression in TCDD-treated fry. 98

Figure 3.26 BAP induction of GFP expression in selected Tg(*cyp1a:gfp*) lines. 99

Figure 3.27 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of mercury chloride. 102

Figure 3.28 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of cadmium chloride. 103

Figure 3.29 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of sodium arsenate. 104

Figure 3.30 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentration of 4-nitrophenol. 107

Figure 3.31 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentration of BPA. 108

Figure 3.32 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentration of TCDD. 109

Figure 3.33 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of lindane. 110-111

Figure 3.34 Mercury exposure of adult Tg(*hsp70:gfp*) fish. 114-115

Figure 3.35 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of 3-MC. 120

Figure 3.36 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of BAP. 121

Figure 3.37 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of TCDD. 122

Figure 3.38	Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of BPA.	124
Figure 3.39	Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of mercury chloride	125
Figure 3.40	Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of lindane.	126
Figure 3.41	Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of 4-nitrophenol.	127
Figure 3.42	TCDD treatment of adult Tg(*cyp1a:gfp*) 4.2 fish.	129

List of Common Abbreviations

3-MC	3-methylcholanthrene
Ac/Ds	Activator/Dissociation
AhR	Aryl hydrocarbon receptor
BAP	Benzo(a)pyrene
bp	base pair
BPA	Bisphenol A
cDNA	DNA complementary to RNA
cyp1a	*cytochrome p450 1a*
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin-11-dUTP
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dpf	days post fertilization
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EPA	United States Environmental Protection Agency
EpRE	Electrophile response element
EROD	Ethoxyresorufin-O-deethylase assay
F*n*	*nth* filial generation
GFP	Green fluorescent protein
HSE	Heat shock element
HSF	Heat shock factor
hsp70	*heat shock protein 70*

kb	kilobase
LB	Luria Bertani broth
LM-PCR	Linker-mediated PCR
MRE	Metals response elements
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse-transcription PCR
TCDD	2,3,7,8-tetrachlorodibenzodioxin
WT	Wild type
XRE	Xenobiotic response elements

INTRODUCTION

1. Introduction

1.1 Water pollution and water quality evaluation

Pollution refers to chemicals or other substances in concentrations greater than those occuring under natural conditions and causing environmental harm and threat to human health (Crathorne *et al.*, 2001). With the exponential human population growth and increased industrialization and urbanization, environmental pollution has become one of the major global problems faced by mankind. Water pollution is a major form of pollution in the environment. Anthropogenic activities such as industrial waste dumping, leaching of minerals from mining activities, run-off of pesticides and fertilizer from farming, sewage leakage and oil spillage contributed to the water pollution.

Water pollution has severe effect on human as well as ecosystem. Firstly, polluted water sources are not suitable for human consumption and often lead to illness when consumed. Even minute amount of pollutants, especially persistent organic pollutants (POPs), can bioaccumulate in the organism and biomagnify through aquatic foodwebs (Schwarzenbach *et al.*, 2010). Hence, besides direct consumption of water, ingestion of contaminated seafood can also be harmful to human health. Secondly, toxic concentrations of pollutants can result in high mortality of organisms in the water bodies and subsequently disrupt the balance of ecosystem. The process in which excessive nutrients discharged to water bodies either through surface run-off or leaching into ground water is termed as eutrophication (Mason, 2001). Although nutrients such as nitrogen and phosphorus are generally not toxic, eutrophication stimulates the growth of algae which leads to disruption of the ecosystem balance (Mason, 2001). Harmful cyanobacterial blooms due to nutrient

over-enrichment have occurred in many large and resourceful water bodies in the world, including Lakes Victoria, Africa; Erie US-Canada; Okeechobee, Florida, USA; Taihu, China; Kasumigaura, Japan; the Baltic Sea in Northern Europe; and the Caspian Sea in West Asia (Paerl *et al.*, 2011). Lastly, in addition to its negative ecological and health impacts, these adverse effects of pollution can cause serious economic losses in fisheries industry.

Due to global climate changes and reduction of wetlands area, the amount of freshwater supply has diminished and thus water scarcity poses a crisis for sustainable growth of the world population (World Water Assessment Programme (United Nations) & Unesco, 2009). Hence, there is an increasing public awareness and concerns regarding the water quality. As such, many government water agencies have set up laws and regulations to safeguard the quality of water. For example, Clean Water Act was enacted to establish a structure for regulating discharges of pollutants into the waters and regulating quality standards for surface waters in USA (US Environmental Protection Agency, 2011).

Usually, samplings of sediment, water or organism are performed at the sites of water bodies for analysis. Most often, analytical chemical methods such as chromatography and mass spectrometry are used to determine the presence and the level of chemicals with precision (US Environmental Protection Agency, 2011). However, these analytical methods are generally expensive and slow as the process includes acquisition of samples, transportation to analytical laboratories, sample processing, data collection and data analysis (Carvan *et al.*, 2000). Technicians have to be highly trained for preparing samples with laborious process, operating the expensive equipment properly and analyzing the data generated.

Sometimes, biological samples are used for detection of pollutants since bioaccumulation occurs in organism and it also reflects the bioavailability of the pollutants. It has been long established that contaminants can bioaccumulate in fish up to 100,000 times higher than the environmental levels, depending on fish species, contaminants properties and water chemistry (Carvan *et al.*, 2000). One study has reported that mercury had been found to be more than 40,000 times enriched in the fish muscle as compared to surrounding water (Kannan *et al.*, 1998). Bioavailability, defined as the fraction of the bulk amount of chemical that is present in the sediment or water which can potentially be taken up during the organism's life time into its tissue (Oost *et al.*, 2003), can be affected by many factors. For example, the bioavailability of inorganic pollutants, including heavy metals, is affected by spontaneous chemical reaction such as oxidation/reduction, complexation, adsorption, and precipitation/dissolution (Schwarzenbach *et al.*, 2010) that can occur in the water bodies.

Biological samples are then used for biochemical assays such as ethyoxyresorufin-O-deethylase (EROD), superoxide dismutase (SOD), catalase test and glutathione peroxidase assay to quantify the activity of these defense enzymes that are frequently induced by toxic chemicals (Carvan *et al.*, 2000). Detection of pollutant-induced mRNAs or proteins from tissues can also be performed (Carvan *et al.*, 2000). For example, the levels of choriogenin mRNA and vitellogenin proteins are quantified in male liver or blood samples respectively to evaluate the level of estrogenic substances (Kurauchi *et al.*, 2005). Such bio-analysis normally requires sophisticated equipments and training that are available in the laboratories only. Great care has to be taken in handling of samples, in order to prevent denaturation or proteolysis of tissue. There are also limitations in the interpretation of such data as

various factors like individual variability, physiological, genetic and metabolic factors have considerable effect on the results.

The observation of stream organisms is also used in conjunction with chemical methods as these biotic indicators reflect the true ecological condition of water body. The underlying concept is that certain types of stream animals thrive only under certain water quality conditions, hence when the conditions change, the distribution and abundance of the animals at affected site will change as well. However, baseline study of the ecosystem has to be first established as the reference point. Further interpretation of these biological indicators requires additional measurements of fundamental and associated components of the abiotic environment, including physical measures of habitat and chemical measures of ambient water quality (U.S. Environmental Protection Agency, 2002). Therefore, current approaches have their own limitations and disadvantages.

1.2 Biomarkers in toxicology

For monitoring of aquatic pollution, it is essential to identify early signals or warnings which reflect the adverse response towards environmental toxins. As such, biomarkers are often used as a tool complementary to chemical and ecological analyses used for monitoring (Oost *et al.*, 2003). Biomarkers, generally, are defined as measurements of an interaction between a biological system and a potential hazard (Sanchez & Porcher, 2009). These include measurements of biochemical, cellular, physiological or behavioral variations which are related to exposure or effects of the chemicals (Sanchez & Porcher, 2009). The proposed characteristics of a good candidate biomarker include the following: Firstly, biomarkers should be reliable and easy to detect at low cost. Secondly, biomarkers should be sensitive to the exposure

and effects of pollutants so as to serve as early warnings. Thirdly, biomarkers should have their impacts of confounding factors on their response and baseline data well established so as to distinguish between natural variability and contaminant-induced stress. Lastly, mechanism between the biomarker response and exposure to pollutant, as well as its toxicological significance should be well defined.

However, due to the large range of possible contaminants encountered in present aquatic pollutions, there is not a single biomarker that can adequately predict all of the toxicant perturbations (Oost *et al.*, 2003). Biomarkers can be derived from parameters or proteins/RNAs such as oxidative stress, biotransformation enzymes, stress proteins, reproductive parameters, immunological parameters, genotoxic parameters and physiological parameters (Torres *et al.*, 2008; Sanchez & Porcher, 2009).

1.3 The use of transgenic fish as water sentinel

1.3.1 Advantages of transgenic fish model system

The transgenic technology has been widely used in biotechnology, from generation of genetically modified foods to pharmaceutical proteins. Since it was first applied to fish in mid-1980s, transgenic studies have been carried out in over 35 fish species, half of which are important for aquaculture (Zbikowska, 2003). With the advent of living colour reporter gene e.g. green fluorescent protein (GFP), living color transgenic fish has been widely used in analyses in embryonic development and gene promoter characterization (Gong *et al.*, 2001; Gong & Korzh, 2004; Udvadia & Linney, 2003). In recent years, transgenic fish have also been generated for biomonitoring aquatic contaminants as in vivo surveillance system (Carvan *et al.*, 2000; Lele & Krone, 1996; Zeng *et al.*, 2005). In these biomonitoring fish, they have

been genetically modified to respond to contaminants with easily detectable reporter, based on the principle that certain genes are inducible by certain chemical contaminants. This involves inserting promoter with inducible DNA response element and a reporter gene such as GFP into the genome of the fish to create these transgenic fish.

The usage of transgenic fish as biomonitoring system has its unique advantages over the conventional surveillance methods. As a transgenic fish is a biological system itself, this provides certain advantages such as bioaccumulation and bioavailability over analytical chemistry methods, thus truly reflecting toxicity towards the organism. As physiology of the fish is taken into account, biological effects of toxicants can be studied at the organ level. With the availability of a large number of transgenic fish lines expressing fluorescent proteins in specific or multiple tissues/organs, unknown developmental toxicity in these tissues or organs can be revealed when tested with chemicals. This method is useful to detect subtle effects of chemicals on the organs which are otherwise overlooked. For example, Tg(*nkx2.2a:mEGFP*) zebrafish that expresses GFP in the nervous system showed reduced GFP expression in the spinal cord with increasing E2 concentration, thus suggesting E2 could cause neurotoxicity (Ng *et al.*, 2012). Recently, Tg(*hsp70:egfp*) transgenic zebrafish (Blechinger *et al.*, 2002) induced GFP expression in the olfactory system of fish after cadmium exposure, indicating sensitive stress response in the olfactory cells that was further confirmed by abnormal histopathology and increased cell death in the olfactory system (Matz & Krone, 2007).

Although it is impossible to accurately quantify or identify the suspected pollutants by using transgenic fish as compared to analytical chemistry approaches, ease of detection and simple analysis of data makes it an attractive approach,

particularly when visible GFP is used as a reporter. GFP fluorescence does not require additional reagents to view. Data analysis can be fast as bioaccumulations of many chemicals occur within minutes and GFP expression can be detected within hours (Carvan *et al.*, 2000). It is relatively cheap and easy to maintain the husbandry of the transgenic fish model like zebrafish and medaka, and no sophisticated equipment is needed besides the fluorescent microscope. Furthermore, the technicians do not need to be highly trained in operating fluorescent microscope and analyzing GFP expression. This assay does not require fish sacrifice and allows repeated testing with the same fish after the fish has recovered back to its original state. The initial step of establishing the transgenic line is tedious and long, however, once the stable line is achieved, this system will provide a simple, economical and practical biomonitoring tools for aquatic pollution.

1.3.2 Small fish models as biomonitoring sentinels

Both zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) have emerged as dominant models in the development of biomonitoring transgenic fish because of the well-established transgenic technology in these two freshwater species. The zebrafish is a tropical freshwater fish descending from the family of cyprinids and is found naturally in rivers of south Asia including northen India, northern Pakistan, Bhutan and Nepal (Dahm & Geisler, 2006). The medaka is a freshwater killifish native to Asia, primarily in Japan, Korea and eastern China (Wittbrodt *et al.*, 2002; Masato kinoshita *et al.*, 2009). Both species are popular in transgenesis due to common attributes such as short generation time, high fecundity, feasibility of manipulation and microinjection of transparent embryos, ease of fish husbandry as well as availability of genomic data. Medaka and zebrafish are also among the few fish species recommended by Organisation for Economic Co-operation and Development

(OECD) for toxicity test (OECD, 1992). Currently, fish are widely used models in toxicology (Carvan *et al.*, 2007) and zebrafish embryos have been suggested to be alternative for adult fish in acute toxicity test to reduce the number of fish used experimentally (Scholz *et al.*, 2008). A protocol for toxicity test using zebrafish Danio rerio embryos (DarT) has been developed and has demonstrated reliable correlation with the results from acute adult fish toxicity test (Fraysse *et al.*, 2006; Nagel, 2002). Furthermore, this protocol could be modified to fit the requirements of other species such as medaka and fathead minnow (*Pimephales promelas*) with comparable results to that of zebrafish (Braunbeck *et al.*, 2005).

It is worth noting that medaka provides several additional advantages over zebrafish in water monitoring. Firstly, it is a temperate fish, thus able to survive under a broad range of temperature from 4°C to 40°C. Secondly, medaka is also able to adapt to a wider range of salinity than most other freshwater fish including zebrafish. Hence, its hardiness allows it to adapt to various climates and regional waters. Thirdly, the sex of medaka can be identified both morphologically and genetically (Masato kinoshita *et al.*, 2009), hence providing added advantages when monitoring certain environmental contaminations related to sex hormones. Lastly, a complete transparent strain of medaka, see-through, is available such that its internal organs including brains, kidney and liver can be observed directly by naked eye (Wakamatsu *et al.*, 2001); thus this strain can be used to achieve a higher sensitivity of detection in fluorescent transgenic adult fish.

1.3.3 Examples of biomonitoring transgenic fish

Currently, there are a few stable transgenic zebrafish and medaka fish lines established for detecting certain classes of pollutants. Zebrafish and medaka remain the most popular fish models in such transgenic works. Majority of the biomonitoring

lines have their transgenes consist of a pollutant-inducible promoter and a reporter gene. The promoter, used in transgenic biomonitoring fish, can be isolated from an inducible gene or artificially engineered based on known DNA response elements. Some of the reported transgenic models are summarized in Table 1.1 according to their responsiveness to the types of pollutants, together with a brief description of its fish species and its transgene.

Usually, the promoter is activated by specific classes of contaminants to transcribe the reporter gene. For example, *vitellogenin* gene was significantly induced in male fish liver during the exposure to estrogenic compounds. Vitellogenin mRNA is almost undetectable in male fish in normal and estrogen-free water but is greatly induced in the presence of estrogen, 17β-estradiol (E2) (Tong *et al.*, 2004). Thus Tg(*mvtg1:gfp*) medaka harboring the medaka *vitellogenin* promoter and GFP reporter gene has been established and has been demonstrated to induce GFP in the liver of male fish when exposed to E2 (Zeng *et al.*, 2005).

Popular choices of reporter genes include luciferase and GFP. Some transgenic lines used luciferase reporter gene as the reporter gene activity can be easily quantified by its bioluminescence assay. For example, (Legler *et al.*, 2000) have established an estrogen responsive luciferase reporter zebrafish line and has demonstrated E2 dosage-dependent increase of luciferase activity. However, this requires sacrifice of transgenic organism, additional assay reagents and experimental procedures. In contrast, GFP is often used as reporter gene due to its visual detection and no requirement of additional reagents, thus allowing on-site real-time detection. It is also possible to quantify GFP expression using approaches such as extrapolation based on the relationship between the intensity of GFP fluorescence and reciprocal

calculated exposure time of the color digital cooled charge-coupled device camera (Kurauchi *et al.*, 2005).

Transgenic fish have also been engineered for detection of mutagens. These transgenic lines contain mutational target transgene instead of the conventional promoter-gene inserts. For example, *rpsL* transgenic zebrafish reported by Amanuma *et al.*, 2000 carry shuttle vector plasmid with mutational target gene, *rpsL* (streptomycin-sensitive gene of *Escherichia coli*) together with a kanamycin resistant gene. Winn *et al.*, 2000 have developed a similar mutational target transgenic zebrafish but using *cII* gene from bacteriophage lambda as target gene instead. After exposure to mutagens, the genomic DNA is extracted from such transgenic zebrafish and the plasmid is rescued for analysis of mutation frequency, locus and type of mutation within mutational target transgene. Thus this approach may assist in identifying classes of mutagens as different mutagens have shown to induce different but consistent patterns of mutation in DNA.

Table 1.1 Examples of biomonitoring transgenic fish

Categories of pollutants	Gene promoters	Species of promoter	Reporter Gene	Transgenic model	System	References
Estrogenic compounds	Tandem repeats of ERE	Artificial	Luciferase	Zebrafish	Stable	Legler *et al.*, 2000
Estrogenic compounds	Choriogenin L	Medaka	GFP	Medaka	Stable	Ueno *et al.*, 2004 Salam *et al.*, 2008
Estrogenic compounds	Vitellogenin 1	Medaka	GFP	Medaka	Stable	Zeng *et al.*, 2005
Estrogenic compounds	Choriogenin H	Medaka	GFP	Medaka	Stable	Kurauchi *et al.*, 2005 Kurauchi *et al.*, 2008
Estrogenic compounds	Vitellogenin 1	Zebrafish	GFP	Zebrafish	Stable	Hao *et al.*, 2006
Heavy metals	Heat shock protein 70	Zebrafish	GFP	Zebrafish	Stable	Blechinger *et al.*, 2002
Heavy metals	Heat shock protein 70	Human	GFP	Zebrafish	Transient	Seok *et al.*, 2006 Seok *et al.*, 2007
Heavy metals	Glutathione S-transferase α1	Mouse	Luciferase - GFP	Zebrafish	Stable	Kusik *et al.*, 2008
Heavy metals	Heat shock protein 27	Zebrafish	GFP	Zebrafish	Stable	Wu *et al.*, 2008
Heavy metals and POPs	Various promoters with AHRE, EPRE or MRE	Mouse or trout	Luciferase	Zebrafish cell culture	Transient	Carvan *et al.*, 2000
Xenobiotic compounds	Cytochrome p450 1a1	Human	GFP Luciferase	Zebrafish and Zebrafish cell culture	Transient	Mattingly *et al.*, 2001
Xenobiotic compounds	Cytochrome p450 1a1	Human	GFP	Zebrafish cell culture	Transient	Seok *et al.*, 2008
Mutagens	-	-	*rpsL*	Zebrafish	Stable	Amanuma *et al.*, 2000
Mutagens	-	-	*cII* *lacI*	Medaka	Stable	Winn *et al.*, 2000
Mutagens	-	-	*lacZ*	Medaka Mummi-chog	Stable	Winn *et al.*, 2001

1.4 Transcriptional response to environmental chemicals

1.4.1 Pollutant response elements

It has been known that many pollutants can induce changes in gene expression such as thioredoxin and heat shock protein genes (Yang *et al.*, 2007). In our study, we rely on the transcription of biomarker genes to develop biomonitoring transgenic fish. Firstly, as this is detected at transcriptional level, it produces an early and quick response. Secondly, this is not affected by post-transcriptional or feedback regulation which influences the enzymatic activity or protein level. The transcription of genes relies on the response elements and regulatory regions located in the promoter region. Response elements are sequence specific DNA motifs that bind to certain activated transcription factors. Intracellular or extracellular stimuli directly or indirectly liberate activated transcription factors that recognize and bind to response elements to either up-regulate or down-regulate target gene expressions (Carvan *et al.*, 2000). A few classes of response elements have been identified to respond to common aquatic pollutants and these are summarized in Table 1.2.

Table 1.2 Summary of response elements to environmental pollutants

Response element	Consensus sequence 5'-3'	Activating agents
Estrogen response element (ERE)	GGTCANNNTGACC	Estrogenic compounds, chlorinated aromatic hydrocarbons and insecticides
Metal response element (MRE)	TGCRCNC	Heavy metals
Xenobiotic response elements (XRE aka AHRE, DRE)	(T/G)NGCGTG	polycyclic aromatic hydrocarbons, dioxins and halogenated aromatic hydrocarbons
electrophile resonse elements (EPRE aka ARE)	GTGACNNNGC	planar aromatic hydrocarbons, Potent electrophiles(heavy metals, quinones, diphenols)
Glucocorticoid response element (GRE)	GRACANNNTGTYC	Steroids e.g. glucocorticoids androgen, minerlocorticoids and progestins
Heat shock reponse element (HSE)	GAANNTTC	External stress(e.g. high temperature)
Hypoxia response element (HRE)	RCGTG	Low oxygen level
Thyroid hormone response elements (TREs)	AGGTCANNNAGGTCA AGGTCATGACCT TGACCA($_{N4-6}$)AGGTCA	Thyroid - pharmaceutical
Peroxisome proliferator response element (PPRE)	AGGTCANAGGTCA	Peroxisome proliferator receptor ligands e.g. prostaglandins and Non-steroidal anti-inflammatory drugs(NSAID)
Retinoic acid response elements(RAREs)	RGGTCA(N_{0-8}) RGGTCA	Retinoic acid and other retinoids-natural and pharmaceutical
Retinoid X response elements (RXRES)	GGGGTCAAAGGTCA GGGGTCATGGGGTCA	

1.4.2 Heat shock protein 70

1.4.2.1 The role of HSP70 as biomarker

We have identified *hsp70* as one of our candidate biomarkers in this project. Heat shock proteins, HSPs, exist ubiquitously in cells and are well conserved among all eukaryotic species as they are required for cell survival. In unstressed cell, they are involved in the maintenance of protein homeostasis including protein folding, aggregation and trafficking. However when cells are under stress, HSPs are up-regulated to prevent aggregation of incomplete polypeptide and thus protect the cells against proteotoxic effect. There are four major Hsp families, including Hsp90 (82-90 kDa), Hsp70 (68-75 kDa), Hsp60 (58-65 kDa) and the small Hsp family (15-30 kDa). The Hsp70 family is the most conserved, best characterized and the largest of all the Hsp families.

Table 1.3 has listed a few chemicals, especially heavy metals such as cadmium and arsenic that have shown up-regulation of *hsp70* in various aquatic organisms after exposure. The exact mechanism of how heavy metals exposure activates *hsp70* signaling pathway was unclear however it was suspected to be due to cross talk with other signaling pathways (Uenishi *et al.*, 2006). Regardless, *hsp70* has been popularly recommended as biomarker for general pollution.

Table 1.3 List of some chemicals that induced expression of *hsp70* in aquatic organisms.

Chemicals	Organisms	References
Arsenic	*Channa punctatus*	(Roy & Bhattacharya, 2006)
	Pimephales promelas	(Farrell *et al.*, 2011)
	Oncorhynchus mykiss	(Boone & Vijayan, 2002)
B-naphthoflavone	*Gobius niger*	Carnevali and Maradonna 2003
Cadmium	*Gammarus fossarum*	(Schill *et al.*, 2003)
	Fucus serratus *Lemna minor*	(Elyse Ireland *et al.*, 2004)
	Oncorhynchus mykiss	(Boone & Vijayan, 2002)
Copper	*Crassostrea hongkongensis*	(Zhang & Zhang, 2012)
	Oncorhynchus mykiss	(Boone & Vijayan, 2002)
Chromate	*Pimephales promelas*	(Landis & Hughes, 1993)
Diazion	*Pimephales promelas*	(Landis & Hughes, 1993)
Heptachlor	*Homarus americanus*	(Snyder & Mulder, 2001)
Lindane	*Pimephales promelas*	(Landis & Hughes, 1993)
Malachite green	*Crassostrea hongkongensis*	(Zhang & Zhang, 2012)
Manganese	*Paraacentrotus lividus*	(Pinsino *et al.*, 2010)
Mercury	*Gadus morhua*	(Olsvik *et al.*, 2011)
Microcystin	*Cyprinus Carpio*	(Jiang *et al.*, 2012)
Nonylphenol	*Gobiu niger*	(Carnevali & Maradonna, 2003)

1.4.2.2 HSP70 signaling pathway

HSP70 is known to be regulated by transcription factor, heat shock factor (HSF), that binds to the heat shock elements (HSEs). HSE is characterized by array of consensus 5'-nGAAn-3' sequences (Morimoto, 1998; Shamovsky & Nudler, 2008; Bierkens, 2000).Two or more HSFs have been discovered in high eukaryotes, while a single HSF gene is expressed in yeast and Drosophila (Shamovsky & Nudler, 2008). In vertebrates, HSF1 is the major transcriptional factor involved during heat shock (Morimoto, 1998). In unstressed cells, HSF1 exists as a monomeric, non-DNA binding form (Bierkens, 2000; Morimoto, 1998). There are a few proposed mechanisms of HSF in activation of the transcription of stress responsive genes. One prevalent hypothesis is that molecular chaperones such as Hsp90 play a role in maintaining transcriptionally repressed HSF1 (Morimoto, 1998). Under stress conditions, misfolded proteins in the cell compete for molecular chaperones such as Hsp90, resulting in the release of HSF1 from chaperones. The monomeric HSF1 would then convert to transcriptionally active trimeric form with multiple hyperphosphorylated serine residues. Recently, activation of HSF1 is also found to be facilitated by ribonucleoprotein complex containing translation elongation factor eEF1a and non-coding RNA called HSR1 (Shamovsky *et al.*, 2006). It is postulated that HSR1 could behave as thermosensor via heat-induced change in conformation. Both mechanisms are likely to coexist and not necessary mutually exclusive. Also, in vitro studies demonstrated that HSF1 had the intrinsic ability to form trimeric DNA-binding form in conditions such as heat shock, increased calcium concentrations, H_2O_2 and low pH. This was probably due to the formation of cysteine disulfide bonds between monomers that were facilitated by the aromatics amino acids in the DNA binding domain of HSF1 (Anckar & Sistonen, 2011). Consequently, the activated

trimeric form of HSF1 possesses high affinity for HSE. After localization into nucleus, activated HSF1 binds to HSE repeats on the promoter region to initiate transcription of stress responsive genes (Shamovsky & Nudler, 2008).

1.4.3 Cytochrome P450 1a1

1.4.3.1 The role of CYP1a as biomarker

Cytochrome P450 family of enzymes plays an important role in biotransformation of drugs, carcinogens, steroids hormones, and environmental toxicants. In xenobiotics degradation pathway, the first phase starts with oxygenation by CYP1a1 and CYP1a2 enzymes. It converts the substrate to more polar metabolites for better elimination. However, such metabolites like arene oxide, diolepoxide and other electrophilic reactive species, are more toxic than its parent compounds and can lead to tumor formation if such intermediates are not converted rapidly to the next metabolites.

In human, *CYP1A1* is expressed at low levels in extrahepatic tissues but is highly inducible in the liver and extrahepatic tissues while *CYP1A2* is constitutively expressed in liver and is also inducible. However, most fish such as goldfish, zebrafish, medaka and Japanese eel, except for rainbow trout, are known to have a single copy of *cyp1a* gene (Oh *et al.*, 2009; Yamazaki *et al.*, 2002; Fujii-Kuriyama & Mimura, 2005).

Nevertheless, CYP1a enzymes are highly inducible at both mRNA and protein levels by a range of polycyclic aromatic hydrocarbons (PAHs) and planar halogenated hydrocarbons (PHHs). Table 1.4 shows a list of some compounds, mainly PAHs, which induced the expression of *cyp1a* gene after exposure. Hence it has been used as a biomarker for exposure of aromatic compounds. As such, enzymatic assay that

measured ethoxyresorufin-O-deethylase activity (EROD) is one of the standard methods to determine PAH contamination. The EROD assay determines the rate of Cyp1a-mediated deethylation of the substrate 7- ethoxyresorufin to form the product resorufin. Liver samples are generally extracted from fish exposed to aquatic pollution and analyzed with EROD assay. The results of EROD assay thus indicate the level of Cyp1a enzyme presents in the sample which is likely to be induced due to the exposure of PAHs and PHHs.

Table 1.4 List of some chemicals that induced expression of *cyp1a* in aquatic organisms

Chemicals	Organisms	References
2,3,7,8-tetrachlorodibenzodioxin	*Danio rerio*	Zodrow *et al.*, 2004
		Andreasen *et al.*, 2002
		Bugiak & Weber, 2009
3-methylcholanthrene	*Poeciliopsis lucida*	Lewis *et al.*, 2004
α-naphthoflavone	*Danio rerio*	Bugiak & Weber, 2009
Β-naphthoflavone	*Danio rerio*	Di Bello *et al.*, 2007
Benzo[a]pyrene	*Danio rerio*	Bugiak & Weber, 2009
	Carassius auratus	Oh *et al.*, 2009
Carbofuran	*Tinca tinca*	dos Anjos *et al.*, 2011
Polychlorinated dibeno-p-dioxins and dibenzofurans	*Sparus aurata*	Abalos *et al.*, 2008
Water soluble fractions of oil	*Danio rerio*	dos Anjos *et al.*, 2011

1.4.3.2 CYP1a signaling pathway

The inducible CYP1a family expression is regulated by the aryl hydrocarbon receptor (AhR) signaling pathway. AhR normally exists in dormant state in cytoplasm in association with HSP90, XAP2 and p23 (Fujii-Kuriyama & Mimura, 2005; Kawajiri & Fujii-Kuriyama, 2007; Mandal, 2005). Upon ligand binding, the complexes dissociates, leaving the nuclear leading signal of AhR exposed. This leads to translocation of AhR subunits into nucleus and formation of heterodimer together with Arnt protein (Fujii-Kuriyama & Mimura, 2005; Kawajiri & Fujii-Kuriyama, 2007; Mandal, 2005). Usually toxicity of POPs is mediated mainly through ligand dependent AhR signaling pathway (Kawajiri & Fujii-Kuriyama, 2007), although studies have shown that the activation of AhR could be ligand independent (Zhou *et al.*, 2010). The heterodimer recognizes and binds to XREs, a consensual DNA sequence of 5'-TNGCGTG-3', in the proximal promoter (Mandal, 2005; Fujii-Kuriyama & Mimura, 2005) and chromatin remodeling is then initiated (Kawajiri & Fujii-Kuriyama, 2007; Whitlock, 1999). Other coactivators and general transcription factors such as SRC-1 and p/CIP were also recruited to form transcription complexes prior to transcription of genes such as CYP1a (Mandal, 2005; Grandjean *et al.*, 2010).

1.5 Aims and objectives in this study

Although the usage of transgenic fish to biomonitor water pollution has long been suggested, only a few transgenic fish lines have been so far established for this purpose. The established transgenic medakas for biomonitoring are only limited to estrogenic category, which includes GFP transgenic lines under *chorigenin H*, *chorigenin L* and *vitellogenin* promoters (Ueno *et al.*, 2004; Kurauchi *et al.*, 2005; Zeng *et al.*, 2005). Although the responses of these transgenic medaka lines to

estrogenic compounds have been well established, there is still a lack of information about the feasibility of using transgenic fish to monitor other environmental toxicants. Thus, in the present study, we would like to establish Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) medaka lines, with an aim to detect heavy metals and PAH compounds respectively. Tg(*cyp1a:gfp*) will be of greater interest as no stable line of Tg(*cyp1a:gfp*) has been established in any fish species while several transgenic zebrafish line with heat-shock stress inducible promoters have been reported (Blechinger *et al.*, 2002; Wu *et al.*, 2008).

In developing these transgenic medaka lines, we plan to use Ac/Ds transposon system to aid the efficiency of transgenesis. Although Ac/Ds is one of the first DNA transposons discovered (McClintock, 1951), only recent studies have demonstrated the versatility of Ac/Ds transposon system in other organisms. With high rate of transgenesis in the zebrafish model aided with the Ac/Ds system, we expect the same improved transgenesis to also apply to the medaka species. As there is no literature that reports the use of Ac/Ds transposon in generating transgenic medaka at the time when this project was initiated, we also planned to analyze the effect of Ac/Ds transposon in the generation of transgenic medaka, so as to evaluate the efficiency of Ac/Ds transposon in medaka.

After establishing the stable medaka lines of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*), it is of interest to characterize these transgenic medaka for their responses toward the targeted chemical pollutants. We will determine its inducibility and sensitivity in order to assess their feasibility as biomonitoring sentinels. Our lab has identified five categories of pollutant that is of interest to public health. These include endocrine disruptors, metalloid compounds, organic nitrogen compounds, organo-chlorine compounds as well as polycyclic aromatic hydrocarbons (PAH) and dioxins. Some

common examples are listed in each category in Figure 1.1. Therefore, both transgenic lines will be exposed to one or few chemicals from each category of pollutants. Table 1.5 lists the chemicals that would be used in this study as well as their effects and possible source of contaminations. Six of these chemicals have been listed as EPA priority pollutants, regulated by EPA due to their frequency of occurrence of at least 2.5% and have been produced in significant quantities in the past (United States Environmental Protection Agency, 2012).

Hence, we would like to propose these following objectives in the current study

1. Characterization of *hsp70* and *cyp1a* promoter regions
2. Establishment of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) using Ac/Ds transposon system
3. Evaluation of Ac/Ds transposon system in generation of transgenic line by analyzing
    a. efficiency of germline transmission
    b. features of Ac/Ds system observed during generation of transgenic lines.
4. Characterization of Tg(*hsp70:gfp*) as biomonitoring tools by observing
    a. its specificity towards various chemicals
    b. GFP expression pattern induced
    c. sensitivity by testing with a range of concentration
5. Characterization of Tg(*cyp1a:gfp*) as biomonitoring tool by observing
    a. its specificity towards various chemicals
    b. GFP expression pattern induced
    c. sensitivitiy by testing with a range of concentration

Endocrine disruptor	Organo-chlorine	Organic nitrogen	Inorganic compounds	Polycyclic aromatic hydrocarbons And dioxins
*Bisphenol A Estradiol Diethylstilbestrol	*Lindane Atrazine Heptachlor	*4-nitrophenol 4-Chloroaniline Diethylnitrosoam-ine	*Mercury *Arsenic *Cadmium	*TCDD *Benzo(a)pyrene *3-methylcholanthrene

Figure 1.1 Different chemical classes of environmental pollutants. The boxes at the bottom row represent examples of chemical found in the category mentioned in the above boxes. Those chemical with asterisk were used in this project.

Table 1.5 List of toxicants used in the study.

Chemicals	Potential health hazard	Common source of contamination
Bisphenol A (BPA)	Developmental effects on reproductive organs, heart disease, diabetes	Degradation of products containing BPA, such as ocean-borne plastic trash, plastic lining of canned foods etc.
Lindane	Kidney or liver problems	Runoff/leaching from insecticide used on cattle, lumber and gardens
4-nitrophenol*	Headaches, drowsiness, nausea and cyanosis.	Runoff/leaching from fungicides and insecticides or dye factories
Mercury*	Kidney damage, Neurotoxicity, developmental defects	Erosion of natural deposits; discharge from refineries and factories; runoff from landfills and croplands.
Arsenic*	Skin damage or problems with circulatory systems, and may have increased risk of getting cancer	Erosion of natural deposits; runoff systems, from orchards; runoff from glass & electronics production wastes
Cadmium*	Kidney Damage	Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints
2,3,7,8-tetrachlorodibenzodioxin (TCDD)*	Reproductive difficulties; increased risk of cancer	Emissions from waste incinerations and other combustion; discharge from chemical factories
Benzo(a)pyrene (BAP)*	Reproductive difficulties; increased risk of cancer	Leachings from linings of water storage tanks and distribution lines
3-methylcholanthrene (3-MC)	Increased risk of cancer	Normally used in laboratories to induce tumor formation in lab animals.

*: denotes EPA priority pollutan

MATERIALS AND METHODS

2. Materials and Methods

2.1 Medaka fish care and generation of transgenic medaka

2.1.1 Fish husbandry

Hd-rR medaka strain was obtained from National Institute for Basic Biology, Okazaki, Japan, through the National BioResource Project (NBRP Medaka), Japan (Masato kinoshita *et al.*, 2009). Husbandry of medaka fish was based on (Iwamatsu, 2004) and in compliance with the Institutional Animal Care and Use Committee (IACUC) of National University of Singapore. Fish were kept in the aquarium with a photoperiod of 14 h light, 10 h dark. Fish were fed with brine shrimp (World Aquafeeds, USA) twice a day. Staging of medaka embryos and fry was mainly based on (Iwamatsu, 2004).

2.1.2 Spawning

Adult male and female fish were separated a day before microinjection. In the morning prior to microinjection, the separated male and female were put together, in a ratio of 2:3 as soon as the light was turned on. After about half an hour of spawning, the female fish was caught in the net and the embryos were removed from its abdomen gently into the petri dish with egg water [0.006% v/ w sea salt(Red Sea)].

2.1.3 Microinjection

Before spawning, microinjection system was set up first to ensure early stage injection. The microneedle, pulled to the shape according to (Rembold *et al.*, 2006) , was filled with injection solution. Injection solution consists of 0.1% phenol red, DNA solution with or without mRNA solution. Solution filled microneedle was then attached to the needle holder of air pressure injector (FemtoJet injector, Eppendorf,

Germany) and positioned at about 45° above the stereomicroscope stage. Before microinjection, the embryos collected were put in chilled embryo water to slow down embryonic development. The attaching filaments of the embryos were gently removed by two pairs of forceps to separate the embryos from each other. Viable embryos were then transferred onto 1.5% agarose (in embryo medium) embryo holder plate and arranged into the grooves. The agarose plate was topped up with embryo medium to cover the embryos. The embryo was then oriented such that the cell was facing the microneedle. Suitable pressure setting was adjusted such that pressure was sufficient to inject solution yet retain compensation pressure to prevent retrograde flow of the embryo contents. Microinjection was performed in the short frame of development from stage 1 to stage 2, with injected volume ranging from 0.55 to 1 nl per embryo. The concentration of the plasmid and *Ac* mRNA were all adjusted at the ratio of 1:5. The concentration of the injected plasmid and *Ac* mRNA is listed as follows; pDs(KRT4-EGFP) (10 ng/µl) with *Ac* mRNA (50 ng/µl); pDs(HSP70-EGFP) (7.5 ng/µl) with *Ac* mRNA (37.5 ng/µl); pDs(CYP1a-EGFP) (10 ng/µl) with *Ac* mRNA (50 ng/µl).

2.1.4 Screening for transgenic founders

After microinjection, the injected embryos were incubated at 28 ^{o}C. Only GFP positive embryos (any level of GFP expression as an indicator of successful microinjection) were raised as F0 founders. After these founders reached sexual maturity, they were crossed with wild type medaka for testing of transgene transmission. Usually Tg(*hsp70:gfp*) F1 embryos were screened at 3 dpf for positive transgene transmission after heat shock induction, which was performed by incubating embryos at 37 ^{o}C for 2 hr followed by returning them back to 28 ^{o}C for 4 hr prior to observation of GFP expression. Tg(*cyp1a:gfp*) F1 embryos were screened

by observing GFP expression during embryonic development from 1 dpf till hatching for positive transgene transmission.

2.2 Molecular techniques

2.2.1 Genomic DNA extraction

Larvae or embryos were pooled together and excess embryo media were removed before rapidly frozen in liquid nitrogen. The samples were kept at -80°C for long storage. After thawing on ice, each sample was added with appropriate amount of DNA extraction buffer (10 nM Tris-HCI pH 8.2, 10 mM EDTA, 200 nM NaCl, 0.5% SDS, 10 μg/ml proteinase K) before homogenization. The samples were incubated at 56°C for 2 hours with brief vortexing every 30 minutes. Equal volume of phenol-chloroform (1:1) was mixed with the sample and centrifuged at 13,000 rpm for 10 minutes. Supernatant was then transferred to a new tube. 0.1X of the original sample volume of 3 M sodium acetate (pH 5.2) and 2X of the original sample volume of 100% ethanol were added and mixed prior to incubation at room temperature (RT) for 15 minutes. The samples were then centrifuged at 13,000 rpm for 5 minutes and supernatants were removed. The pellets were washed with 200 μl of 70% ethanol and centrifuged again at 13,000 rpm for 5 minutes. The pellets were air dried after removal of ethanol. Appropriate amount of autoclaved MiliQ water was used to dissolve the DNA pellet before storage at -20 ^{o}C. Concentration of DNA was measured using Nanodrop 2000 (Thermo Fisher Scientific, USA). Depending on downstream applications, RNAse was sometimes added to the samples to remove RNA contamination.

Genomic DNA extraction for adult fish was similar to above with slight modifications. The adult fish was rapidly frozen and pounded in liquid nitrogen. The

pounded sample was usually aliquoted into two or more eppendorf tubes on ice, with each tube filling up to a third of the volume. After addition of 500 ul of DNA extraction buffer, the tubes were incubated at 56 ^{o}C for at least 6 hours with occasional and brief vortexing to ensure complete tissue digestion. Following from there, the rest of the procedure is similar to that of genomic DNA extraction of larvae and embryos.

2.2.2 RNA extraction

Embryos or larvae were pooled together in an eppendorf tube and excess medium was removed. For later processing, the tubes were then rapidly frozen in liquid nitrogen before storage at -80 ^{o}C. Sample was homogenized in 300 µl of TRIzol reagent (Invitrogen, USA) using a power homogenizer before addition of remaining 700 µl of TRIzol. The homogenized tissue was incubated at room temperature for 5 minutes to allow for complete dissociation of nucleoprotein complexes. Each tube was added with 0.2 ml of chloroform and shaken vigorously for 15 seconds. Following incubation at room temperature for 3 minutes, samples were centrifuged at 12,000 x g for 15 minutes at 4 °C to allow the mixture to separate into a lower phenol-chloroform phase, an interphase and an upper aqueous phase. As RNA remains exclusively in the aqueous phase, this layer was transferred into a fresh eppendorf tube and care was taken not to disrupt the interphase layer to prevent contamination with genomic DNA. To precipitate RNA from the isolated aqueous phase, 0.5 ml of isopropyl alcohol was added and incubated at room temperature for 10 minutes, before centrifugation at 12,000 x g for 10 minutes to collect the precipitated RNA as a gel-like pellet. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol and vortexed briefly to ensure complete washing. To re-collect the pellet, the tube was centrifuged at 7,500 x g for 5 minutes

at 4 °C before the supernatant (75% ethanol) was discarded. Pellet was then air-dried for not more than 10 minutes, before being dissolved in diethyl pyrocarbonate (DEPC)-treated water. RNA concentration was then measured by Nanodrop 2000 (Thermo Fisher Scientific, USA). Integrity of the RNA was evaluated with RNA gel electrophoresis and UV spectrophotometry. A260/A280 ratio of 1.8-2.0 indicates high quality RNA. For long term storage, RNA was kept at -80°C.

2.2.3 Polymerase chain reaction (PCR)

Standard PCR reaction was performed using GoTaq Flexi DNA polymerase kit (Promega, USA) with a total reaction volume of 10 µl consisting of 2 µl of 5X Green GoTaq Flexi Buffer, 0.4 µl of 25mN magnesium chloride solution, 1 µl of dNTP mixture (2 mM ATP; 2 mM GTP; 2 mM CTP; 2 mM TTP), 0.25 µl of 10 uM forward primer, 0.25 µl of 10 uM reverse primer, 0.2 µl of Taq polymerase (5 units/µl) and 0.5 to 1 µl of template DNA, with addition of MilliQ water adjusted accordingly to final volume of 10 µl.

A typical PCR reaction was set up with the cycling conditions as follows: initialization at 95 ^{o}C for 5 minutes; 25 to 30 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 55 to 62 ^{o}C for 1 minute, extension at 72 ^{o}C for 45-90 seconds; final extension at 72 ^{o}C for 10 minutes. All PCR products were analyzed on 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, USA) and visualized using an ultraviolet transilluminator in a gel documentation system Gel Doc XR (Bio-Rad Laboratories, USA). Table 2.1 shows the list of primers that was used in this project for PCR reactions.

Table 2.1 List of primers used for various PCR reactions.

Primer	Sequence 5'- 3'	Amplified Targets
HSP70 XhoI	CCCTCGAGGTCTCTGTGATGGTCTACT	*hsp70* promoter
HSP70 EcoRI	GGAATTCGGTTTGCTGGATGACTTTG	
CYP1a EcoRI	GGAATTCGAGGACAATGACAGCAGGATCT	Proximal *cyp1a* promoter
CYP1a NcoI	GATGCCATGGGTAAATTAGATC	
DisCYP1a Xhol	CCTCGAGGCACCTCCGTGCAGGCTGTG	Distal *cyp1a* promoter
DisCYP1a EcoRI	CGGAATTCGATCCTGCTGTCATTGTCCTC	
GFP probe F	AAGGGCGAGGAGCTGTTCAC	*gfp* probe for Southern blot
GFP probe R	CTTCTCGTTGGGGTCTTTGC	
CYP1a1 DIG F	GAGGACAATGACAGCAGGAT	*cyp1a* promoter probe for Southern blot
CYP1a1 DIG R	GTAAATTAGATCACTGAAAACACC	
HSP70 DIG probe F	AGCTGGGACTGACAGAAGGA	*hsp70* promoter probe for Southern blot
HSP70 DIG probe R	CGCTTTATATCTGCGGAAGC	
QRT-CYPF	CCCAAAGACACGTGTGTCTT	*cyp1a* cDNA for in situ hybridization
QRT-CYPR	GCATCAATGCCTGTAATGCC	
HSP-RTF	CACAAAGTCATCCAGCAAAC	*hsp70* cDNA for in situ hybridization
realt-HSP R	TCAGTCCACCTCCTCAATAG	

2.2.4 One step reverse transcription PCR (RT-PCR)

RNA samples in the same set of experiment were diluted to the same concentration before performing reverse transcription PCR with QIAGEN OneStep RT-PCR kit (QIAGEN, Germany). The total volume for each reaction was 25 µl, including 5 µl of 5X RT-PCR buffer, 1 µl of dNTP mix (10 nM), 1 µl of one-step RT-PCR enzyme mix, 1 µl of RNA template and 0.25 µl of reverse and forward primer (10 µM) each, topped up with autoclaved MiliQ water. Reverse transcription PCR reaction was carried out at 50 ℃ for 30 minutes and followed by a standard PCR reaction setting.

2.2.5 Synthesis of *Ac* mRNA for microinjection

The pAc-SP6 plasmid was digested with BamHI, and gel purified to obtain linearised DNA. The linearised DNA was used to generate capped mRNA in vitro using the mMessage mMachine SP6 kit (Ambion, USA) by following the manufacturer's protocol with slight modification – the transcription was incubated at 30 ^{o}C for 3 hours instead. The resultant capped RNA was precipitated by lithium chloride method. Briefly, 1 µl of 0.5M EDTA pH 8.0, 2.5 µl of 4M lithium chloride and 75 µl of 100% cold ethanol were added to the mixture and left to precipitate in -20 ^{o}C for at least 30 minutes. The mixture was centrifuged at 4°C for 15 min at 14,000 rpm to pellet the RNA. Supernatant was carefully removed and the pellet was washed with 500 µl of 70% cold ethanol. Again, the mixture was centrifuged at 4 ^{o}C at 14,000 rpm for 10 minutes. The ethanol was decanted and the pellet was air-dried for about 5 minutes before being dissolved in DEPC-treated water.

2.2.6 Whole mount *in situ* hybridizations

2.2.6.1 Synthesis of DIG labeled RNA probe for *in situ* hybridizations

10 μg of plasmid DNA carrying the gene of interest was cut at the 5' end using the appropriate restriction enzyme for 2 hours at 37 °C. Complete digestion was confirmed through running a small amount of the digestion product in 1% agarose gel. Upon confirmation of complete linearization, the remaining product was subjected to PCR purification using QIAQUICK PCR Purification Kit (Qiagen, Germany) as according to manufacturer's instructions.

1 μg of linearized DNA was used as template for in vitro transcription of RNA probe. The reaction was carried out in a total volume of 10 μl consisting of 1 μg linearized DNA, 2 μl of 5X reaction buffer, 4 μl of DIG-RNA labelling mix (Roche, Germany) [10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP and 3.5 mM DIG-UTP], 1 μl Protector RNase inhibitor (40 U/μl) (Roche, Germany) and 1 μl of appropriate RNA polymerase (T7, T3, or SP6; Ambion, USA). The reaction mix was incubated at 37 ºC for 1.5 hours, followed by digestion with DNase I (Ambion, USA) for 15 minutes at 37 °C to remove DNA template. The product was purified using the RNA cleanup protocol from the RNEasy Mini Kit (Qiagen, Germany) according to manufacturer's instruction. The RNA was finally eluted using 30 μl of RNase-free water and kept at -20 °C until further use.

2.2.6.2 Fixation and proteinase K digestion of the embryos

Medaka embryos or larvae were fixed in 4% paraformaldehyde (PFA) in 2X PBS (0.8% NaCl, 0.02% KCl, 0.0144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) at room temperature for 4 hours or at 4 ^{o}C overnight on a nutator (CLAY ADAMS® brand, Becton Dickinson, USA). Following that, embryos were dechorionated with sharp

needles carefully. The steps performed are at room temperature on a nutator otherwise indicated else. The samples were washed with PBST (0.1% Tween®20, PBS) 4 times for 5 minutes each. The PBST was then replaced with 100% methanol for 5 minutes, followed with new replacement of 100% methanol for storage at - 20°C to allow better penetration of probes.

Gradual rehydration of the samples were performed with subsequent changing of the medium with 75% methanol in PBS, 50% methanol in PBS, 25% methanol in PBS and finally PBST for 5 minutes each time. The samples were washed with PBST again for 5 minutes and digested with proteinase K (10 µg/ml PBS) with very gentle shaking. Time of proteinase K exposure was adjusted according to the age of embryo. For 2 dph embryos (stage 22 to 24), proteinase K digestion period was 5 minutes. As for 1 dpf fry, the period of proteinase K digestion was 25 minutes to prevent over digestion of epithelial tissue, or 75 minutes to have better probe penetration into internal organs. To stop the digestion, proteinase K was removed and replaced with freshly prepared glycine (2mg/ml PBS) briefly twice before fixation with 4% PFA/1XPBS for 20 minutes. The samples were then washed with PBST for 5 minutes 5 times.

2.2.6.3 Hybridization of DIG probes

The following steps were performed in water bath preheated at 65°C unless indicated otherwise. The samples were prehybridized for 2 hours in Hyb-Mix [50% formamide, 5X SSC (150mM NaCI, 15mM sodium citrate, pH 7.0), 50 µg/ml heparin, 5 mg/ml tRNA, 0.1% Tween®20, pH 6.0] . In the meantime, DIG-labelled RNA probes were diluted at 1:20 in hybridization buffer and subjected to denaturation by heating at 80 °C for 10 minutes followed by 5 minutes of ice bath. The original buffer

of the samples was then gently removed and replaced with denatured probe for hybridization overnight.

Following hybridization, probe was removed and replaced with prewarmed 50% formamide in 2X SSCT [2X SSC, 0.1% Tween®20] and incubated for 30 minutes twice. The embryos were subsequently subjected to the following washes: 2X SSCT for 15 minutes, 0.2XSSCT for 30 minutes twice. The embryos were then washed with malate buffer (100 mM maleic acid,150 mM NaCI, 0.1% Tween®20) for 15 minutes at room temperature.

2.2.6.4 Antibody incubation and staining

Blocking was performed by incubating the embryos in 2% blocking solution (Blocking reagent, Roche, Germany) in malate buffer for two hours at room temperature to block non-specific antibody binding sites. Blocking solution was then removed and samples were incubated with anti-DIG-AP antibody at 1:2000 dilution in blocking solution on a nutator at 4°C overnight.

After overnight antibody incubation, the samples were washed with PBST 6 times for 10 minutes each on nutator at room temperature. To equilibrate the samples, the solution was replaced with freshly made staining buffer (100 mM TrisCl, pH 9.5, 100 mM NaCI, 50 mM MgCl2, 0.1% Tween®20) for 5 minutes on a nutator twice. 4.5 µl of NBT (Nitroblue Tetrazolium, Boehringer Mannheim, Germany; 50 mg/ml in 70% dimethyl formamide) and 3.5 µl of BCIP (5-bromo, 4-chloro, 3-indolylphosphate, Boehringer Mannheim, Germany; 50 mg/ml in water) were added into 1 ml staining buffer with embryos, mixed carefully, and incubated in dark at room temperature for a few minutes to several hours to develop staining. Progress of the staining was continuously monitored using a stereomicroscope. After staining has developed to

desired intensity, embryos were washed in PBST thrice for 5 minutes each before long storage in 50% glycerol in PBS. The images of in situ hybridization were taken using stereomicroscope Olympus MVX10 with digital camera Olympus DP72 (Olympus, Japan).

2.3 Plasmid Constructs

2.3.1 Restriction endonuclease digestion of DNA

Digestion reactions were usually performed at 37 ^{o}C for 2 hours. Typically, 5 μg of plasmid was used for restriction enzyme (RE) digestion. The reaction volume consisted of 5 μl of 10X buffer, 2 μl of restriction enzyme, 0.5 μl of bovine serum albumin, variable amount of DNA template and topped up with MiliQ water to total volume of 50 μl. To stop the reaction, the mixture was placed at 65 ^{o}C for 10 minutes.

2.3.2 Ligation

Ligation reactions were carried out using T4 DNA ligase kit (New England Biolabs, USA). The reaction consisted of 1 μl of 10X ligation buffer (0.3 M Tris-HCI, pH7.8; 0.1 M DDT and 5 mM ATP), 1 μl of T4 DNA ligase, variable volumes of vector DNA, insert DNA and topped up with MilliQ water up to 10 μl of total reaction volume. Vector DNA and insert DNA were added according to a molar ratio of approximately 1:3. The mixtures were either incubated at 16 ^{o}C overnight or at room temperature for 20 minutes.

2.3.3 Transformation and Retransformation

An Eppendorf tube containing 100μl of DH5α competent *Esherichia coli* bacteria was first thawed on ice before adding 5 μl of ligation product. The mixture was then incubated on ice for about 10 minutes before subjecting to heat shock at 42

^{o}C for 90 seconds. Immediately after heat shock, the tube was placed on ice for additional 5 minutes, followed by addition of 500 µl of Luria Bertani (LB) (Invitrogen, USA) broth. The tube was then incubated at 37 ^{o}C for 1 hour with constant agitation at 250 rpm. After incubation, the transformed *E. Coli* was spun down at 4,000 rpm for 5 minutes and excess LB solution was decanted leaving about 100 µl of LB solution in the tube. The bacterial was resuspended again in 100 µl of LB solution before spreading onto an LB agar plate containing appropriate antibiotics (30 µg/ml kanamycin or 100 µg/ml ampicillin) using glass beads. The LB agar plate was then incubated at 37 ^{o}C for 16 hours.

Regarding retransformation of bacteria for amplification of a plasmid, similar procedures were performed with slight modifications. Instead of 5 µl of ligation products, 0.5 to 1µl of plasmid (100 ng/µl) was added to the thawed competent *E. coli*. In brief, the *E. coli* was then heat shocked and incubated at 37 ^{o}C for 1 hour with similar conditions as mentioned above. 100 µl of the retransformed *E.coli* were directly spread on a LB agar plate containing appropriate antibiotics (30 µg/ml Kanamycin or 100 µg/ml Ampicillin) using glass beads. The LB agar plate was incubated at 37 ^{o}C for 16 hours.

2.3.4 Colony screening

To verify successful ligation and transformation of bacteria, colony screening PCR was performed. Standard PCR ingredients with the exception of DNA template were first prepared as described in Section 2.2.3. The two primers used in colony screening were such that one was designed to anneal to the vector DNA and the other was designed to anneal to the insert DNA. This was to identify the presence of insert in the correct orientation. Transformed colonies were picked with white sterile pipette tips and spotted on another LB agar plate with appropriate antibiotics (30 µg/ml

Kanamycin or 100 μg/ml Ampicillin) , followed by pipetting the colonies into the prepared PCR mixtures. The colonies were spotted in the same order as the PCR reactions so that desired colonies can be identified from the positive results of PCR. Colony screening PCR was performed while the LB agar plate was incubated at 37 ^{o}C for at least 4 hours to ensure sufficient growth of the colony.

2.3.5 Plasmid amplification and purification

After selecting the colonies that contained the desired inserts in the right orientation, the colonies were then inoculated into 15 ml bacterial culture tubes not containing more than 5 ml of antibiotic-containing LB broth. The tubes were incubated at 37 ^{o}C overnight with constant agitation at 250 rpm. After overnight growth, the culture was centrifuged at 4,000 rpm for 5 minutes, and the supernatant decanted. The plasmid from the culture pellet was then isolated and purified with Wizard Plus SV Miniprep DNA purification System (Promega, USA) according to the manufacturer's instruction. In the final step, 50 μl of autoclaved MilliQ water was added to the spin column and centrifuged at 14,000 rpm for 1 minute to elute the plasmid DNA. The plasmid DNA was then quantified with Nanodrop 2000 (Thermo Fisher Scientific,USA) and sequencing was performed to further confirm the insert sequence.

2.3.6 DNA Sequencing

Sequencing of DNA was carried out by automated sequencing using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The sequencing reaction was carried out in a total volume of 20 μl, consisting of 8 μl Terminator Ready Reaction Mix, 200 ng of double-stranded DNA, and 1 μl primer (0.2 μg/μl). PCR was performed with 25 cycles of 96 °C for 10

seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes, and finally hold at 4 °C. Ethanol precipitation was carried out to purify the extension products. 2 µl of 3 M NaOAc (pH 4.6) and 50 µl of 95% ethanol was mixed with the reaction and incubated at room temperature for 15 minutes. The tube was centrifuged at 4 °C for 20 minutes at 14,000 rpm. The pellet was rinsed with 250 µl of 70% ethanol and air-dried before sending for sequencing using ABI 3730xl DNA analyser (Applied biosystems, USA). Sequence obtained was then further analysed and aligned using software Vector NTI Suite 8 (Invitrogen, USA).

2.3.7 DNA vectors

2.3.7.1 pDs(KRT4-EGFP)

Fragment of KRT4-EGFP was purified from double digestion of p(KRT4-EGFP) with XhoI and NotI, using gel extraction kit according to manufacturer's instruction. pDsLFABPGRASv12G which contains the minDs element vector backbone was kindly given to us by Dr Sergui Parinov, Temasek Life Sciences. pDsLFABPGRASv12G was double digested with XhoI and NotI, followed by gel extraction purification of vector backbone that contained minDs (pDs vector). Both digested KRT4-EGFP fragment and pDs vector backbone were ligated together to form the pDs(KRT4-EGFP).

2.3.7.2 pDs(HSP70-EGFP)

The *hsp70* promoter was amplified in PCR using primers, HSP70 XhoI and HSP70 EcoRI, that have additional XhoI and EcoRI restriction site. The genomic DNA was used as DNA template for the PCR. Subsequently, PCR fragment was digested with XhoI and EcoRI, followed by ligation into XhoI/EcoRI digested

pEGFP-1 vector to form pHSP70-EGFP. The hsp70-egfp cassette from pHSP70-EGFP was then subcloned into pDs vector to form pDs(HSP70-EGFP).

2.3.7.3 pDs(CYP1a-EGFP)

The *cyp1a* promoter was amplified in two parts as the full 2.2-kb length was difficult to amplify by PCR perhaps due to AT repeats region. Hence proximal *cyp1a* promoter was amplified in PCR using primers, CYP1a EcoRI and CYP1a NcoI, that have additional EcoRI and NcoI restriction site. On the other hand, distal *cyp1a* promoter was amplified in PCR using primers, DisCYP1a XhoI and DisCYP1a EcoRI, that have additional XhoI and EcoRI restriction sites. The genomic DNA was used as DNA template for both PCR. Subsequently, both PCR fragments were ligated together with EcoRI digested ends of both distal and proximal cyp1a promoter. The final ligated PCR fragment was then purified with gel extraction and cloned into XhoI/NcoI cut pEGFP-1 vector to form p(CYP1a-EGFP). After which, the cyp1a-egfp cassette from pCYP1a-EGFP was then subcloned into pDS vector to form pDs(CYP1a-EGFP).

2.4 Analysis of genomic insertions

2.4.1 Southern blot analysis

2.4.1.1 Synthesis of DIG-labeled DNA probe

DIG-Nick Translation Kit (Roche, Germany) was used for the synthesis of DIG-labeled DNA probes for Southern blot analysis. 4 µl of DIG-Nick Translation mix was added to an Eppendorf tube containing 1 µg of template PCR purified products in 16 µl of sterile water that was prechilled on the ice. The ingredients were mixed and spun down before incubating at 15 ^{o}C for 90 min. To stop the reaction, 1 µl of 0.5 M EDTA, pH 8.0 was added to the tube before heating at 65 ^{o}C for 10 min. The

purification of DIG probe was performed using lithium chloride method as previously described in Section 2.2.5. The pellet was dissolved in TE buffer (10 mM Tris-HCI, 1mM EDTA, pH 8.0) and concentration was measured using Nanodrop 2000 (Thermo Fisher Scientific, USA).

2.4.1.2 DNA digestion and Separation

10 µg of genomic DNA sample was digested with NdeI (New England Biolabs, USA) and MfeI (New England Biolabs, USA) overnight at 37 ^{o}C with similar composition as described in Section 2.3.1. The digestion was inactivated at 65 ^{o}C for 20 minutes before separation of DNA on 0.8% agarose gel electrophoresis at constant voltage, 100 volt for 1 hour and 20 minutes. The gel was then stained with SYBR® Safe DNA gel stain (Invitrogen, USA) with gentle shaking for 5 minutes and viewed in Gel Doc XR system (Bio-Rad Laboratories, USA).

2.4.1.3 Southern blot transfer

After staining, DNA gel was submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl) with gentle shaking for 15 minutes at room temperature twice before rinsing with sterile, distilled water. The gel was then submerged in neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 minutes at room temperature twice. Following that, the gel was equilibrated in 20X SSC for at least 10 minutes before setting up the blot transfer according to a manual from Roche, Germany. It was allowed to transfer overnight in 20X SSC. After that, DNA on the blot membrane is fixed by UV crosslinking at 120 mJ with UV Stratalinker 1800 (Stratagene, USA). Subsequently, the membrane was rinsed briefly in sterile distilled water and air dried.

2.4.1.4 Southern blot hybridization

The membrane was prehybridized with prewarmed DIG Easy Hyb (Roche, Germany) for at least 30 minutes with agitation at hybridization temperature. The hybridization temperature was set at 45°C for *hsp70* and c*yp1a* probe while it was set at 50°C for *gfp* probe. The probe was first denatured at 100 ^{o}C in 50 µl of water for 5 minutes before addition to DIG Easy Hyb to constitute hybridization buffer (27-50 ng/ml). After prehybridization, the DIG Easy Hyb was replaced by hybridization buffer and incubated at the hybridization temperature with agitation for overnight. The membrane was then washed with low stringency buffer [2X SSC, 0.1% sodium dodecyl sulfate (SDS)] for 5 min at room temperature twice before washing with prewarmed high stringency buffer (0.1X SSC, 0.1% SDS) for 15 minutes at 68 ^{o}C twice.

2.4.1.5 Antibody incubation of Southern blot

The following steps were carried out at room temperature and with agitation using the reagents from DIG Wash and Block Buffer set (Roche, Germany). The blot was washed with washing buffer for 2 minutes, followed by blocking with blocking solution for 30 minutes. The solution was then replaced with anti-DIG alkaline phosphatase (Roche, Germany) solution at 1:10,000 dilution in blocking solution for 30 minute incubation. To wash off excess antibody, the blot was incubated with washing buffer for 15 minutes twice. After that, the blot was equilibrated with Detection Buffer for 3 minutes before adding CDP-star (Roche, Germany) evenly. After 5 minutes of addition, the membrane was exposed to X-ray film Amersham Hyperfilm ECL (GE Healthcare, UK) with optimized exposure settings.

2.4.1.6 Stripping of probe

To reprobe the membrane with another probe, firstly the membrane had to be rinsed with sterile distilled water soon after detection. Following that, the membrane was incubated with the stripping buffer (0.2 M NaOH, 0.1% SDS) for 15 minutes at 37°C twice before rinsing with 2X SSC for 5 minutes at room temperature. The membrane was either stored in 2X SSC at 4 °C or processed with another probe as described in Section 2.4.1.4.

2.4.2 Linker-mediated PCR (LM-PCR)

The protocol was adapted from (Wu *et al.*, 2003) . The upper strand linker oligonucleotide was annealed to the lower strand linker oligonucleotide to make the double stranded Hsp92II linker. Genomic DNA was first digested with Hsp92II (Promega) at 37 °C for 4 hours prior to ligation overnight at 16 °C with Hsp92II linker. LM-PCR was performed with primer specific to the linker (linker primer) and the other primer specific to either min3' Ds (Tail-Ds3-1) or min5'Ds (Tail-Ds5-1), using the PCR conditions described here: initialization at 95 °C for 5 minutes; 30 cycles of denaturation at 95 °C for 15 seconds, annealing at 59 °C for 30 seconds, extension at 72 °C for 1 minute; final extension at 72 °C for 2 minutes. 1μl of PCR products was further used for nesting PCR with the nested primers- linker primer nest, Tail-Ds3-2, Tail-Ds5-2. The PCR products were further enhanced with secondary nesting PCR with the secondary nested primers –linker primer nest 2, Tail-Ds3-3 and Tail-Ds5-3. The first PCR was performed using PFU ultra hotstart enzyme (Stratagene,USA) , while the nested PCR was performed using GoTaq HotStart polymerase enzyme (Promega, USA), with composition according to the manufacturer's instructions. The PCR products were then cloned into pGEM-T Easy vector (Promega, USA) to

facilitate sequencing of the PCR products. The primers used in LM-PCR are listed in Table 2.2.

Table 2.2 List of Primers used in LM-PCR

Primer	5'-3' sequence
Upper strand Linker	GTAATAGCACTCACTATAGGGCTCCGCTTAAGGGACATG
Lower strand Linker	PO_4-TCCCTTAAGCGGAG-NH_2
Linker Primer	GTAATAGCACTCACTATAGGGC
Tail-Ds3-1	CGATTACCGTATTTATCCCGTTCG
Tail-Ds5-1	CCGTTTACCGTTTTGTATATCCCG
Linker Primer nest	AGGGCTCCGCTTAAGGGAC
Tail-Ds3-2	CCGGTATATCCCGTTTTCG
Tail-Ds5-2	AATCGGTTATACGATAACGGTCG
Linker Primer nest2	GGCTCCGCTTAAGGGACATG
Tail-Ds3-3	CCCGTTTTCGTTTCCGTCC
Tail-Ds5-3	CGGTCGGTACGGGATTTTCC

2.4.3 Basic Local Alignment Search Tools (BLAST) analysis

Flanking sequences that were obtained from LM-PCR were analyzed in Vector NTI Suite 8 (Invitrogen, USA). The flanking sequences were aligned with the sequence of min Ds element to isolate the genomic flanking sequence. Typically the genomic flanking sequence from min Ds 3’ and min Ds 5’ end of each line was aligned by their 8 bp duplication site and the whole sequence was BLAST against the *Oryzias latipes* Hd-rR strain genomic DNA database in Ensembl release 65 (Dec 2011) . The setting was set as search against DNA database <LatestGp> using search tool <BLASTN> with search sensitivity set as <Near-exact matches>.

2.5 Treatments of the transgenic embryos and adult

2.5.1 Chemicals stock solution preparation

Chemicals used for exposure were obtained from Sigma-Aldrich. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo[a]pyrene (BAP), 3-methylcholanthrene (3-MC), bisphenol A (BPA) and lindane were dissolved or diluted in dimethyl sulfoxide (DMSO) while 4-nitrophenol, mercury(II) chloride, cadmium chloride and sodium arsenate dibasic heptahydrate were dissolved in MiliQ water. For every working concentration used, 1000X concentrated stock solution was prepared. This was to ensure that each final concentration of chemical exposure would have the same amount of vehicle solvent. However, as sodium arsenate has low solubility in water, the stock solutions of sodium arsenate were 100X concentrated instead. All chemical stock solutions were kept at 4 ^{o}C and in dark.

2.5.2 Chemical exposure treatment

2.5.2.1 Chemical exposure treatment of larvae

5 µl of the stock solution of the chemical was diluted in 5 ml of embryo medium in each of the 6-well plate with the exception of arsenic chloride. For arsenic, 500 µl of the arsenic chloride stock solution was diluted in 4.5 ml of embryo medium water. 1-3 dph hemizygous transgenic fry were transferred from a petri dish, with Pasteur pipette to each well of the 6-well plate with minimal carry over of embryo medium. Each well contained the same number of fry, from 5 to 10 fry. As there would be no feeding for the fry, preliminary exposures were terminated after 5 days of treatment as the fry would most likely die after 7 days of starvation. Every day, dead fry (no heart beat) were taken out and disposed. Changing of medium was performed every one or two days by transferring the fry to another plate with the same chemical concentrations.

2.5.2.2 Chemical exposure treatment of adult fish

Tg(*hsp70:gfp*) 1.1 hemizygous transgenic fish were exposed to mercuric chloride concentrations of 0.1 mg/l, 0.2 mg/l and 0.4 mg/l and water as control for a period of 5 days while Tg(*cyp1a:gfp*) 4.2 hemizygous transgenic fish were exposed to TCDD concentrations of 32.2 ng/l, 161.0 ng/l and 805 ng/l and DMSO 0.1% as vehicle solvent control for a period of 3 days. In each experiment, the fish were first acclimatized the day before the start of the treatment. 5 male and 5 female of 6 months old transgenic fish were kept together in plastic tank (24x13x13 cm) with 3 liters of dechlorinated water with adjusted final chemical concentrations. Two female and two male fish were randomly taken from each tank to examine for GFP expression on 1st, 3rd and 5th day. The solution was changed every two days. Any dead

fish was taken out and disposed every day. The fish were not fed during the period of chemical exposures. At the end of each experiment, all fish were checked for GFP expression.

2.5.3 Heat shock treatment

No more than 15 embryos of either 2 dpf or 3 dpf embryos were placed in an Eppendorf tube containing 500 µl of embryo medium. The Eppendorf tube was then incubated at 37 ^{o}C water bath for 2 hours for heat shock. The control batch was placed at 28 ^{o}C for 2 hours. After that, the embryos were then placed back in petri dish at 28 ^{o}C for another 2 hours before observation with a fluorescent microscope.

2.5.4 Fluorescence and image capture

Fry or adult fish were anesthetized in 0.1% phenoxyethanol for 1 to 2 minute to immobilize it before GFP fluorescence observation. To position the embryo or fry for image capture, it was pipetted into 3% methyl cellulose on a petri dish with minimal amount of water. Embryos and fry were observed under an inverted fluorescence microscope (Axiovert 200M, Zeiss) equipped with a digital camera (Axiocam HRc, Zeiss) for capturing GFP expression. As for adult fish, GFP expression was observed and captured on a stereomicroscope, Olympus MVX10 with digital camera Olympus (DP72) (Olympus, Japan).

RESULTS

3. Results

3.1 Characterization of two inducible medaka promoters

3.1.1 Inducible expression of *hsp70* and *cyp1a* mRNAs in medaka embryos.

hsp70 and *cyp1a* are two well known inducible genes and biomarkers for environmental pollution. Numerous studies have shown that *hsp70* is one of the few robust biomarkers that arises after environmental insults by heavy metal and several other chemicals (Mukhopadhyay *et al.*, 2003; Bierkens, 2000). As for *cyp1a*, it remains the most prominent biomarkers for detecting persistent organic pollutants such as PCB and PAH. Therefore, we selected the two genes for their pollutant-inducible promoters in order to develop biomonitoring transgenic medaka.

The medaka genome from an inbred medaka strain, Hd-rR, has been completely sequenced (Kobayashi & Takeda, 2008) and the genome sequence is available in public databases in Ensembl Genome Browser as well as in National Center for Biotechnology Information (NCBI). We found 5' upstream and transcript sequences of medaka *hsp70-1* (called *hsp70* in this study for simplicity), Ensembl gene ID ENSORLG00000000233, and *cyp1a*, Ensembl gene ID ENSORLG00000014421, by searching the Ensembl genome browser database (Ensembl 51, Nov 2008).

3.1.1.1 Up-regulation of *hsp70* by heat shock and mercury treatment.

PCR primers (Table 2.1) were designed based on *hsp70* cDNA sequence in order to determine the level of *hsp70* mRNA via reverse transcription PCR. *hsp70* mRNA was shown to be induced by heat shock treatment at 37 ^{o}C as compared to the control group at 28 ^{o}C (Fig. 3.1A). *hsp70* mRNA was also induced in mercury-

treated embryos in a dosage-dependent manner (Fig. 3.1B). Thus, *hsp70* was inducible by both heat shock and mercury treatment.

3.1.1.2 Up-regulation of *cyp1a* by TCDD and BAP treatments

Medaka *cyp1a* cDNA had been described by Kim et al. (Kim *et al.*, 2004). In the present study, *cyp1a* PCR primers (Table 2.1) were designed based on its cDNA sequences in order to determine *cyp1a* transcript level by reverse transcription PCR. *cyp1a* mRNA was shown to increase by TCDD (1.7 µg/l) compared to DMSO (0.1%), vehicle solvent control (Fig. 3.2A). Similarly, *cyp1a* transcript was also significantly increased in the presence of ≥160 µg/l of BAP (Fig. 3.2B). Thus, *cyp1a* was up-regulated by both BAP and TCDD.

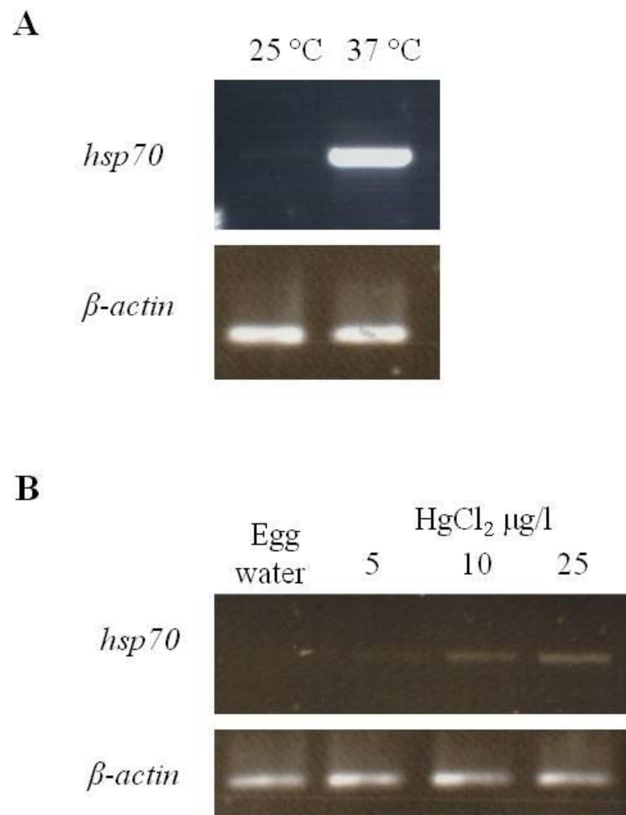
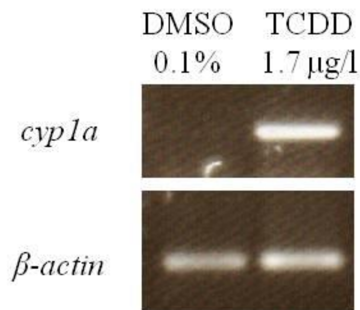


Figure 3.1 Induction of *hsp70* mRNA by heat shock (A) and mercury (B). RNA was extracted from pooled embryos and analyzed by RT-PCR. The level of *β-actin* transcript was used as loading control. (A) Increase of *hsp70* mRNA under heat shock treatment of 6-dpf embryos at 37 ^{o}C for 2 hours. (B) Dosage dependent increase of *hsp70* mRNA in $HgCl_2$-treated of 6-hpf embryos for 7 days.

A



B

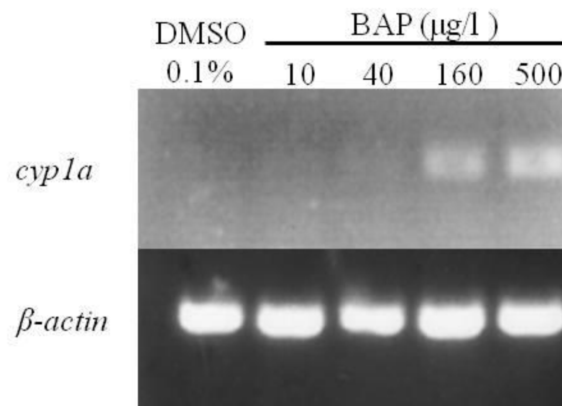


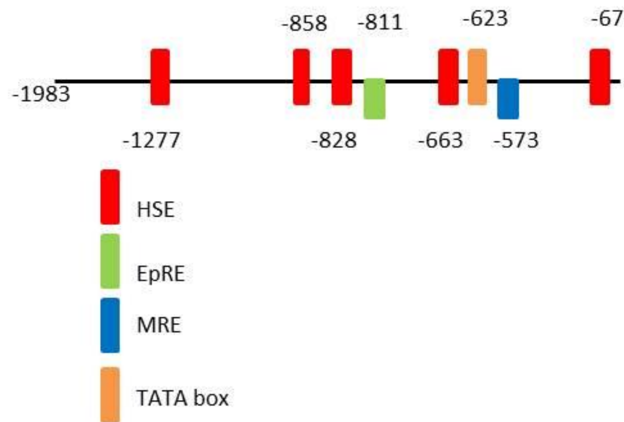
Figure 3.2 Induction of *cyp1a* mRNA by TCDD (A) and BAP (B). RNA was extracted from treated embryos and analyzed by RT-PCR. The level of *β-actin* transcript was used as loading control. (A) Increased in *cyp1a* mRNA under TCDD treatment of 6-dpf embryos for 3 days. (B) Dosage dependent increase of *cyp1a* mRNA in BAP-treated of 3-dpf embryos for 3 days.

3.1.2 Analysis of response elements in promoter region

3.1.2.1 *hsp70* promoter and hsp70-EGFP construct

Medaka *hsp70* promoter sequence was located from the Ensembl genome browser database. Sequence analysis indicated that the basal transcription factor binding region, the TATA box, was located at -623 bp upstream from the translation start codon. Since the minimal sequence required for HSF1 trimer binding includes two DNA consensus sequence -nGAAn in head-to-tail orientation (Shamovsky & Nudler, 2008), five such putative heat shock elements (HSEs) were identified within 2 kb from the start codon (Fig. 3.3A). Therefore, the 2-kb *hsp70* promoter should contain sufficient HSEs for heat shock induction. In addition, one putative metal response element (MRE) and one putative electrophile response element (EpRE) were also identified within 2 kb (Fig. 3.3A). *hsp70* promoter was cloned from -1983 to -1 upstream of the start codon, linked with EGFP cDNA and subsequently cloned into the plasmid vector between two Ds terminal repeats (Fig. 3.3B). The construct, named pDs(HSP70-EGFP), was then sequenced and confirmed by alignment with sequences obtained from Ensembl using Vector NT1 AlignX. The cloned *hsp70* promoter sequence from pDs(HSP70-EGFP) was 99.0% identical to that from Ensembl genome browser database.

A



B

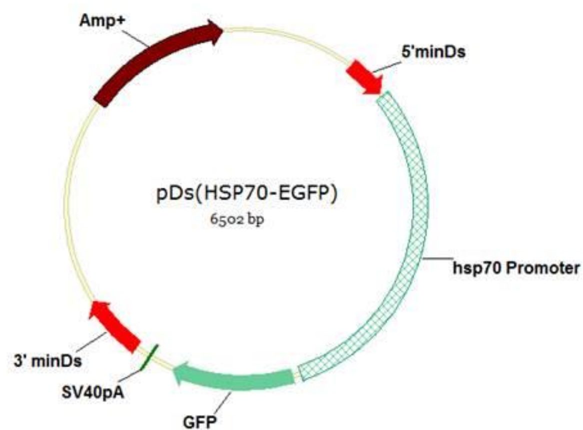
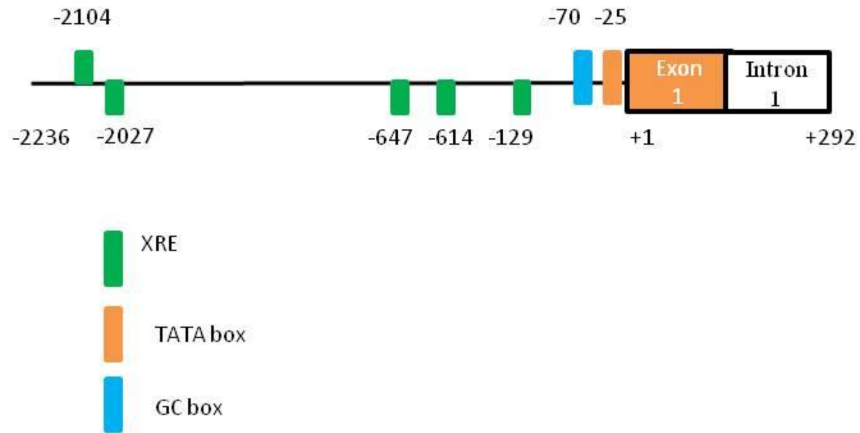


Figure 3.3 Schematic diagram of *hsp70* promoter (A) and *hsp70*-GFP construct, pDs(HSP70-EGFP) (B). In (A), red, green, blue and orange boxes indicate the position of heat shock element (HSE), electrophile response element (EpRE), metal response element (MSE) and TATA box (TATAA) respectively. Position here indicates the nucleotides relative to the translation start codon of *hsp70-1*. Boxes at the bottom of the line represent negative orientation while those on top represent positive orientation, else the box at both sides represent palindrome sequence. In (B), *hsp70* promoter in (A) is linked to EGFP cDNA, with insertion between two minDs elements of the pDs plasmid backbone.

3.1.2.2 *cyp1a* promoter and cyp1a-EGFP construct

Medaka *cyp1a* promoter sequence was also located from Ensembl genome browser database. TATA box and GC box were identified at -25 and -70 bp upstream of the transcription start site of *cyp1a* gene. Three xenobiotic response element (XRE) was found in the proximal region less than 1 kb away from the transcription start site (Fig. 3.4A). The next upstream XRE cluster, consisting of two XREs at -2027 and -2104, were about 1.4 kb upstream of the proximal XRE cluster. Functional analysis of regulatory elements in both eel (Ogino *et al.*, 1999) and zebrafish (Zeruth & Pollenz, 2007) *cyp1a* promoters had demonstrated that the distal clusters of XRE is vital in transcription efficiency. Therefore, the *cyp1a* promoter cloned here included two distal XREs (-2027, -2104) (Fig. 3.4A). Since exon 1 of *cyp1a* gene covers 5' untranslated region only, the promoter fragment was cloned further downstream til exon 2 in order not to miss any regulatory elements in exon1 and intron 1. Therefore, the promoter was amplified by PCR from -2236 to +292, to construct pDs(CYP1a-EGFP) (Fig. 3.4B). The construct was sequenced and confirmed by alignment with sequences obtained from Ensembl using Vector NT1 AlignX. The cloned *cyp1a* promoter sequence from pDs(CYP1a-EGFP) was 96.9% identical to those from Ensembl genome browser database.

A



B

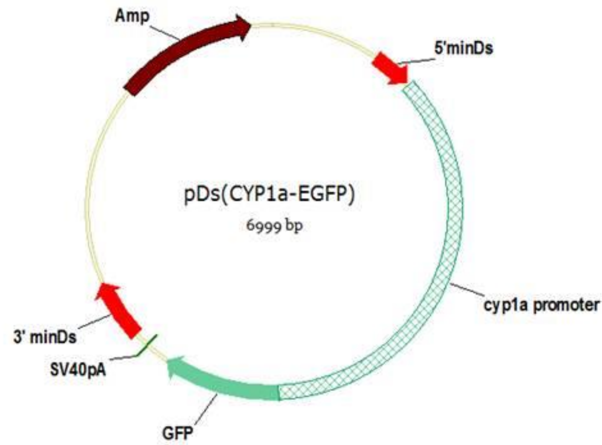


Figure 3.4 Schematic diagram of *cyp1a* promoter (A) and *cyp1a*-GFP construct, pDs(CYP1a-EGFP) (B). In (A), green, orange and blue boxes indicate the position of xenobiotics response element (XRE). TATA box (TATAA) and Sp1 site (GC). Position here indicates the nucleotides relative to the transcription start codon of *cyp1a*. Boxes at the bottom of the line represent negative orientation while those on top represent positive orientation, else the box at both sides represent palindrome sequence. In (B), *cyp1a* promoter in (A) is linked to EGFP cDNA, with insertion between two minDs elements of the pDs plasmid backbone.

3.1.3 Transient transgenic analyses

3.1.3.1 Induction of GFP expression of pDs(HSP70-EGFP)- injected embryos by heat shock and mercury

pDs(HSP70-EGFP) was injected together with *Ac* mRNA into medaka embryos and the injected embryos of 2 dpf were subjected to heat shock treatment for 2 hours and return to 28 ^{o}C thereafter. Strong GFP expressions were induced ubiquitously in the embryonic body in all of the injected embryos that were subjected to heat shock (Fig. 3.5C, D) while no or weak GFP expression was observed in the non-heat shock group (Fig 3.5A, B). The pDs(HSP70-EGFP)-injected embryos of 3 dpf were also treated with mercury chloride of 200 μg/l. Three out of nine injected embryos displayed highly elevated GFP expression in embryonic body and yolk layer after 72 hours of exposure (Fig. 3.6C, D), as compared to the non-treated group (Fig. 3.6A, B) where none of the embryos showed high GFP expression. Perhaps due to the individual differences during the microinjection of the plasmid, those microinjected embryos with higher transgene insertions were able to induce GFP noticeably upon mercuric chloride exposure while others showed insignificant GFP increase or none. Slight GFP expression in the yolk and epithelial cells were sometimes present in some of the injected embryos prior to both heat shock (Fig. 3.5A, B) and mercury chloride (Fig. 3.6A, B) treatments, possibly due to ectopic expression of transgene sometimes observed in microinjected embryos (Rocha *et al.*, 2004). However, tremendous increase of GFP expressions observed in both heat shock and mercury treatment groups signified that the pDs(HSP70-EGFP) was functional and inducible by these factors.

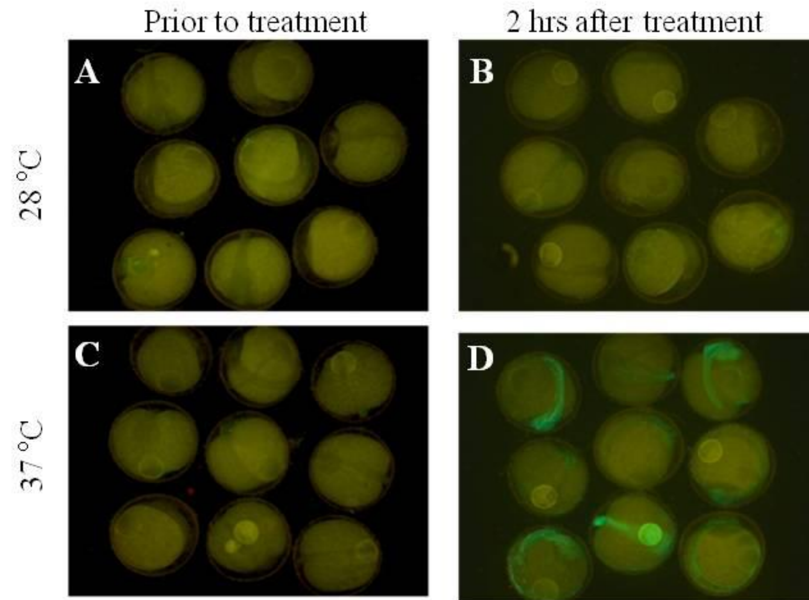


Figure 3.5 Transient GFP expression of pDs(HSP70-EGFP)-injected 2-dpf embryos under heat shock. Images of embryos before and after incubation at 28 ^{o}C (A,B) or at 37 ^{o}C (heat shock) (C,D).

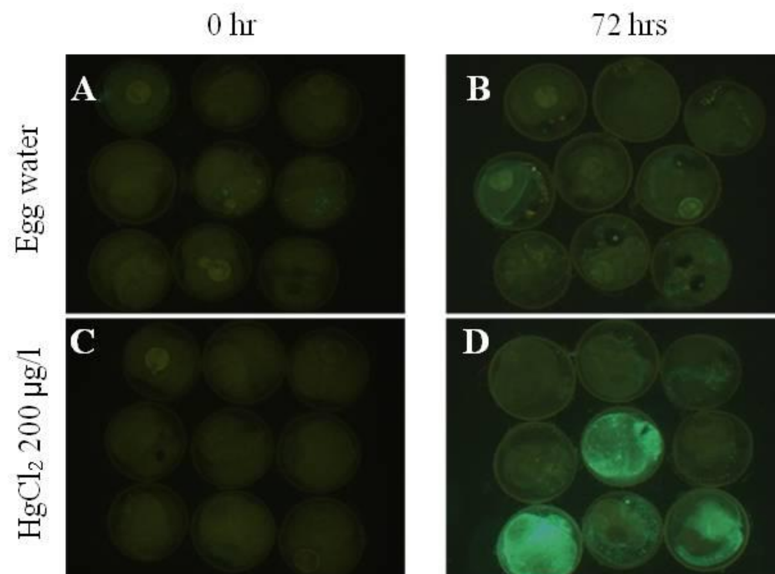


Figure 3.6 Transient GFP expression of pDs(HSP70-EGFP)-injected 3-dpf embryos induced by mercury chloride. Images of embryos before and after exposure to embryo medium control (A,B) and mercury chloride (C,D).

3.1.3.2 Induction of GFP expression in pDs(CYP1a-EGFP)-injected embryos by BAP and TCDD treatment

To assess the functionality of the plasmid, pDs(CYP1a-EGFP), it was injected together with *Ac* mRNA into the embryos which were subjected to treatment with known *cyp1a* inducers, BAP and TCDD. Moderate constitutive GFP signals were observed mainly in yolk in many of the microinjected embryos at 3 dpf (Fig. 3.7A, C and Fig. 3.8A, C). The constitutive expression persisted throughout development to 6 dpf as observed in the control groups for both BAP (Fig. 3.7B) and TCDD (Fig. 3.8B) treatments. GFP expression was not significantly increased in BAP-treated group (Fig. 3.7D) as compared to pre-treatment (Fig. 3.7C) and control group (Fig. 3.7B). Similarly, GFP expression was not significantly increased in the TCDD-treated group either (Fig. 3.8D) as compared to pre-treatment (Fig. 3.8C) and control group (Fig. 3.8B). Thus, the inducibility of the *cyp1a* promoter is not apparent in the transient assay. However, according to our experience in transgenic expression, a more reliable demonstration of promoter validity would be by the development of stable transgenic lines.

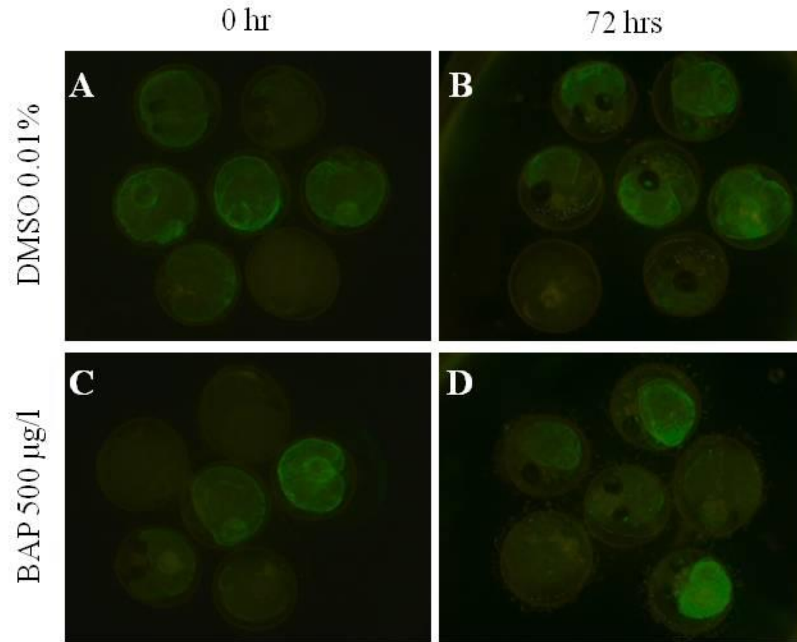


Figure 3.7 Transient GFP expression of pDs(CYP1a-EGFP)-injected 3-dpf embryos induced by BAP. Images of embryos before and after exposure to vehicle control (A,B) and BAP (C,D).

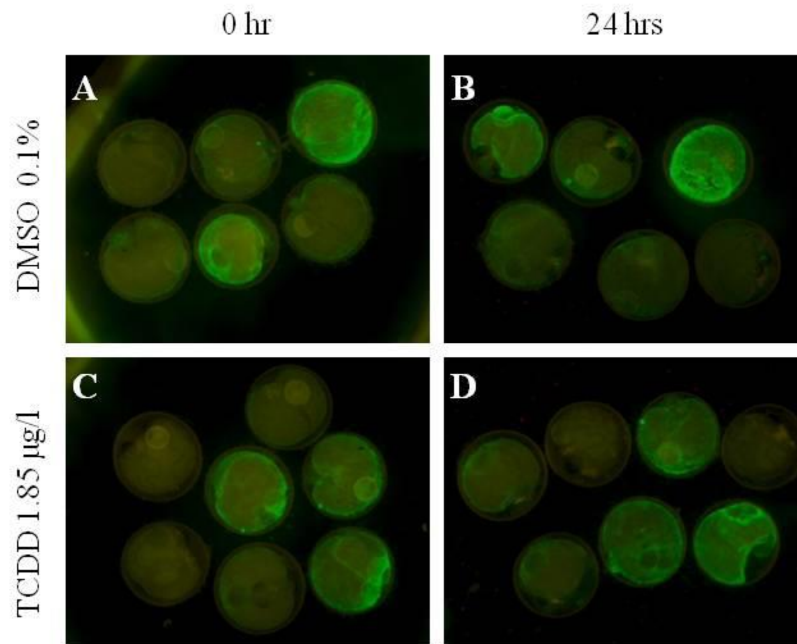


Figure 3.8 Transient GFP expression of pDs(CYP1a-EGFP)-injected 3-dpf embryos induced by TCDD. Images of embryos before and after exposure to vehicle control (A,B) and TCDD (C,D).

3.2 Using the maize Ac/Ds transposon to develop transgenic medaka

3.2.1 Enhanced transient transgenic expression using maize Ac/Ds transposon in medaka.

To determine whether the maize Ac/Ds system can improve transgenesis in medaka, pDs(KRT4-EGFP) was injected into the embryos together with *Ac* mRNA. This plasmid construct contains a well characterized zebrafish promoter *krt4* which has skin epithelial specificity in zebrafish (Ju *et al.*, 1999; Gong *et al.*, 2002) and is also faithfully functional in transgenic medaka (Zeng *et al.*, 2005). Since GFP expression was restricted to skin epithelia in early embryos after injection of pDs(KRT4-EGFP), it was relatively easy to estimate the number of GFP-expressing cells for semi-quantitative analysis. Thus, embryos were classified into four categories based on number of GFP-expressing cells at 3 dpf after microinjection of pDs(KRT4-EGFP) with or without *Ac* mRNA: 0 cell, <10 cells, 10 cells-50% epithelial surface, and >50% epithelial surface, as exampled in Fig. 3.9A-D respectively. The intensity and epithelial area of GFP expressions were obviously higher in the group co-injected with *Ac* mRNA (Fig. 3.9E) than in the group without *Ac* mRNA (Fig. 3.9F). This was further supported by the statistics of the two groups of injected embryos based on the above four categories of GFP expression (Fig. 3.9G). For example, 32.7% of the embryos co-injected with *Ac* mRNA showed GFP expression >50% epithelial surface while only 3.4% of the injected embryos without *Ac* mRNA was classified into this category.

Similarly, another construct pDs(HSP70-EGFP) was microinjected into the embryos with or without *Ac* mRNA. GFP expressions were categorized into three groups: no GFP expression (-), patchy expression (+) and ubiquitous expression (++),

as exampled in Fig. 3.10A-C respectively. In the group of embryos co-injected with *Ac* mRNA, there was a drastic increase, from 10% to 63%, in the percentage of embryos with ubiquitous expression after heat shock (Fig. 3.10D). In comparison, ubiquitous expression was only observed in 6.3% of injected embryos without *Ac* mRNA after heat shock (Fig. 3.10D). The total increase in percentage of embryos that had induced GFP expression including patchy and ubiquitous expression after heat shock treatment was also higher in *Ac* mRNA-injected group (56.6%) than in the other group (50%).

Thus, the maize Ac/Ds system clearly enhanced GFP reporter expression in the transient transgenic system by testing the two different GFP constructs: one under the constitutive *krt4* promoter and the other under the inducible *hsp70* promoter.

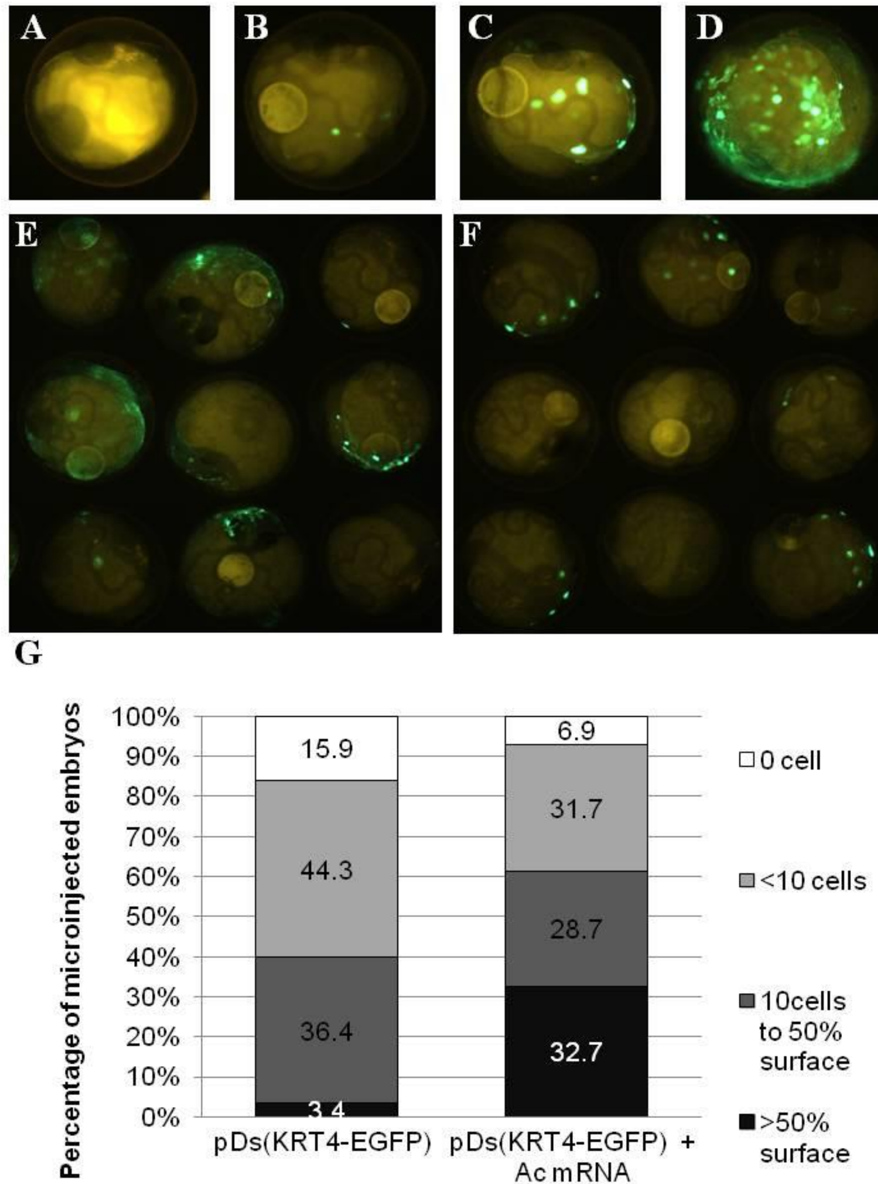
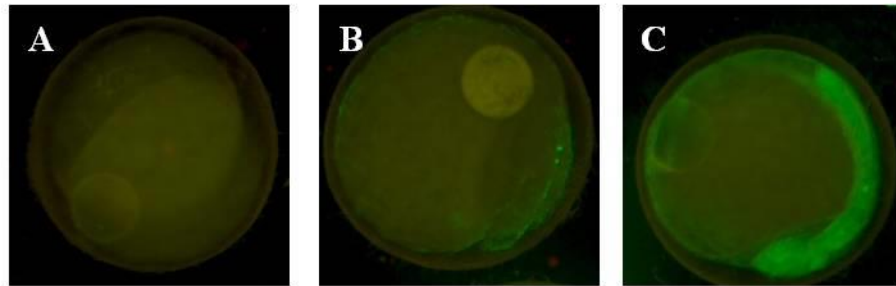
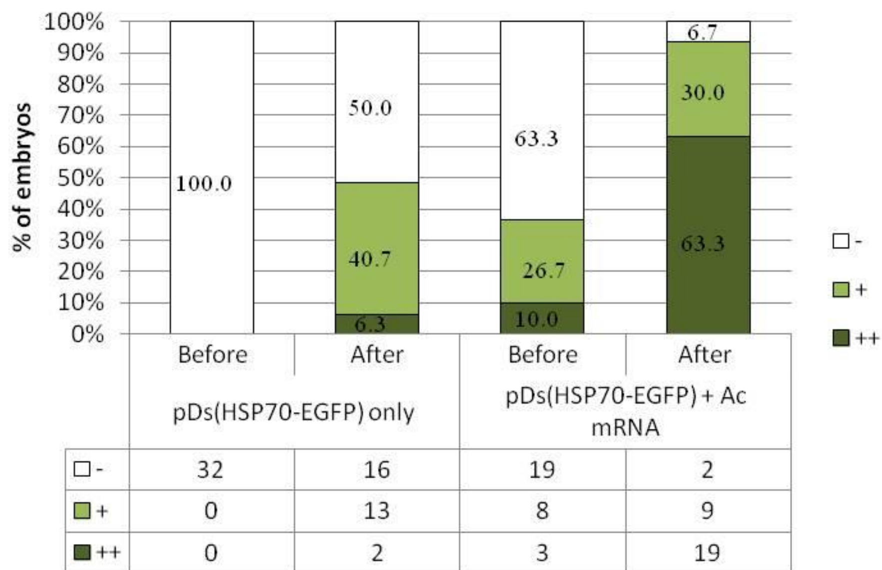


Figure 3.9 Analysis of transient GFP reporter expression using Ac/Ds transposons system with pDs(KRT4-EGFP). (A-D) The level of GFP expression of microinjected embryos was based on the number of GFP-expressing epithelial cells: 0 cells (A), <10 cells (B), 10 cells to 50% epithelial surface (C), >50% of epithelial surface (D). (E,F) Representative images of 6 dpf wild type embryos were microinjected with pDs(KRT4-EGFP) together with (E) or without (F) *Ac* mRNA. (G) Histogram of the percentages of embryos expressing GFP as shown in (A-D) without or with *Ac* mRNA. n=88 and 101 for microinjection of pDs(KRT4-GFP) without and with *Ac* mRNA respectively.



D



	pDs(HSP70-EGFP) only		pDs(HSP70-EGFP) + Ac mRNA	
	Before	After	Before	After
□ -	32	16	19	2
+	0	13	8	9
++	0	2	3	19

Figure 3.10 Analysis of transient GFP reporter expression using Ac/Ds transposons system with pDs(HSP70-EGFP). (A-C) The level of GFP expression observed in pDs(HSP70-EGFP)-injected 2-dpf embryos after heat shock treatment is categorized as no expression (-) (A), weak or patchy expression (+) (B), ubitquitious expression (++) (C) in embryonic body. (D) Histogram of percentage of microinjected embryos expressing GFP in category (A-C) before and after heat shock treatment. There are two groups of embryos that were microinjected with pDs(HSP70-GFP) with or without *Ac* mRNA. Numbers of embryos in each group were presented in the table below.

3.2.2 Generations of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) transgenic medaka

After demonstration of enhanced GFP expression by co-injection of *Ac* mRNA, which was likely due to a more efficient early integration of injected DNA into genome aided by the Ac/Ds transposon system, we injected *Ac* mRNA together with pDs(HSP70-EGFP) or pDs(CYP1a-EGFP) into medaka embryos in order to establish stable Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) medaka lines.

In the group of embryos injected with pDs(HSP70-EGFP), about 71.0% (22/31) of them expressed GFP in various parts of the body and they were raised to maturity. Out of these F0 fish, 12 fish were screened for germline transmission by subjecting their F1 embryos to heat shock treatment. After heat shock screening, 10 out of 12 fish (83.3%, or 59.1% without GFP prescreening) were confirmed of positive GFP transgenic progenies with frequencies ranging from 2.9% to 100.0% (Table 3.1).

In the pDs(CYP1a-EGFP)-injected group, 88.9% (16/18) of injected embryos showed weak GFP expression in the yolk as well as embryonic epithelial cells. The GFP expressing embryos were raised to adult. All 4 F0 fish screened (100%, or 88.9% without GFP prescreening) produced GFP-expressing F1 progeny with frequencies from 24.4% to 88.2% (Table 3.1).

Thus high germline transmission rates (83.3%-100%) were achieved by using Ac/Ds transposon system. Even if considering the transmission rates using total injected embryos, 59.1% and 88.9% from pDs(HSP70-EGFP) and pDs(CYP1a-EGFP) microinjection respectively were quite high compared to typical germline transmission rates (<10%) without using a transposon system in transgenic medaka (Kinoshita *et al.*, 2000; Miyamoto *et al.*, 2009; Zeng *et al.*, 2005; Zeng *et al.*, 2005).

Table 3.1 Frequency of transgenic progeny of positive pDs(HSP70-EGFP) and pDs(CYP1a-EGFP) founders

Construct	Founder fish	Sex	GFP+ embryos	Total no. of embryos	Percentage of GFP+ (%)
pDs(HSP70-EGFP)	1	♀	125	125	100.0
	2	♀	36	147	24.5
	3	♂	85	128	66.4
	4	♂	93	107	86.9
	5	♂	18	104	17.3
	6	♂	2	70	2.9
	7	♂	9	77	11.7
	8	♂	22	64	34.4
	9	♀	2	64	3.1
	10	♂	6	87	6.9
	11	♀	0	92	0.0
	12	♂	0	103	0.0
pDs(CYP1a-GFP)	1	♀	79	135	58.5
	2	♂	34	59	57.6
	3	♀	21	86	24.4
	4	♂	67	76	88.2

3.2.3 Variable constitutive and induction GFP expression in F1 generation

In all the transgenic F1 embryos from all founders of Tg(*hsp70:gfp*), GFP expression was observed in the lens from 3 dpf and remained constitutively thereafter. Besides the lens expression, some transgenic embryos also expressed GFP in the muscle with variable intensity even within the same batch of embryos from the same transgenic founder (Fig. 3.11A). Since *hsp70* was up-regulated by heat shock treatment in medaka embryos, Tg(*hsp70:gfp*) was subjected to heat shock to examine its inducibility of transgenic expression. After heat shock, bright and ubiquitous GFP fluorescence was observed in all transgenic embryos (Fig. 3.11B). Apparently, GFP expression in the lens was a convenient marker for identifying transgenic offspring as no GFP expression was induced by heat shock from those lacking lens-GFP expression.

Different patterns of constitutive GFP expressions (Fig. 3.12A-E) were also observed among the same batch of F1 Tg(*cyp1a:gfp*) fry derived from the same transgenic founder. For example, the transgenic fry seen in Fig. 3.12C had GFP expression in vertebra, lens, yolks, lips and weakly in muscle while fry in Fig. 3.12E had GFP expression in gills and epithelial skin cells around abdomen and sometimes in lens. Multiple GFP phenotypes were also observed in other three Tg(*cyp1a:gfp*) families (Fig. 3.12F-I). Each founder family had seven or more distinct phenotypes with different combinations of GFP expression in various organs. Although GFP expressions across the four founder families were similar, there were certain phenotypes observed only in specific founder families. For example, GFP expression in vertebra was observed only in founder 4 family (Fig. 3.12C).

As *cyp1a* promoter was inducible by several xenobiotic chemicals including TCDD, we treated the F1 fry with TCDD (1.83µg/l) to check for inducibility of

transgene. 24 hours after TCDD exposure, strong and robust GFP expression was induced generally in the liver, intestine and kidney (Fig. 3.12J-M) in all the transgenic fry of the four founder families tested, irrespective of variable constitutive GFP expressions (Fig. 3.12F-I).

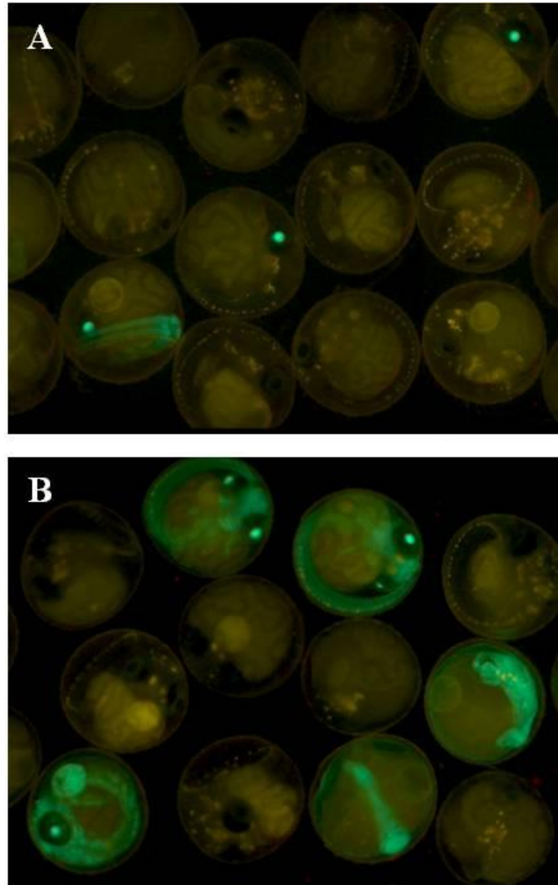


Figure 3.11 Heat shock inducible GFP expression in 6-dpf F1 embryos of Tg(*hsp70:gfp*). (A,B) Representative images before (A) and after heat shock treatment (B). Note that all transgenic embryos had GFP expression in lens and could be induced to express GFP throughout embryos after heat shock. Non-transgenic siblings had no GFP expression at all.

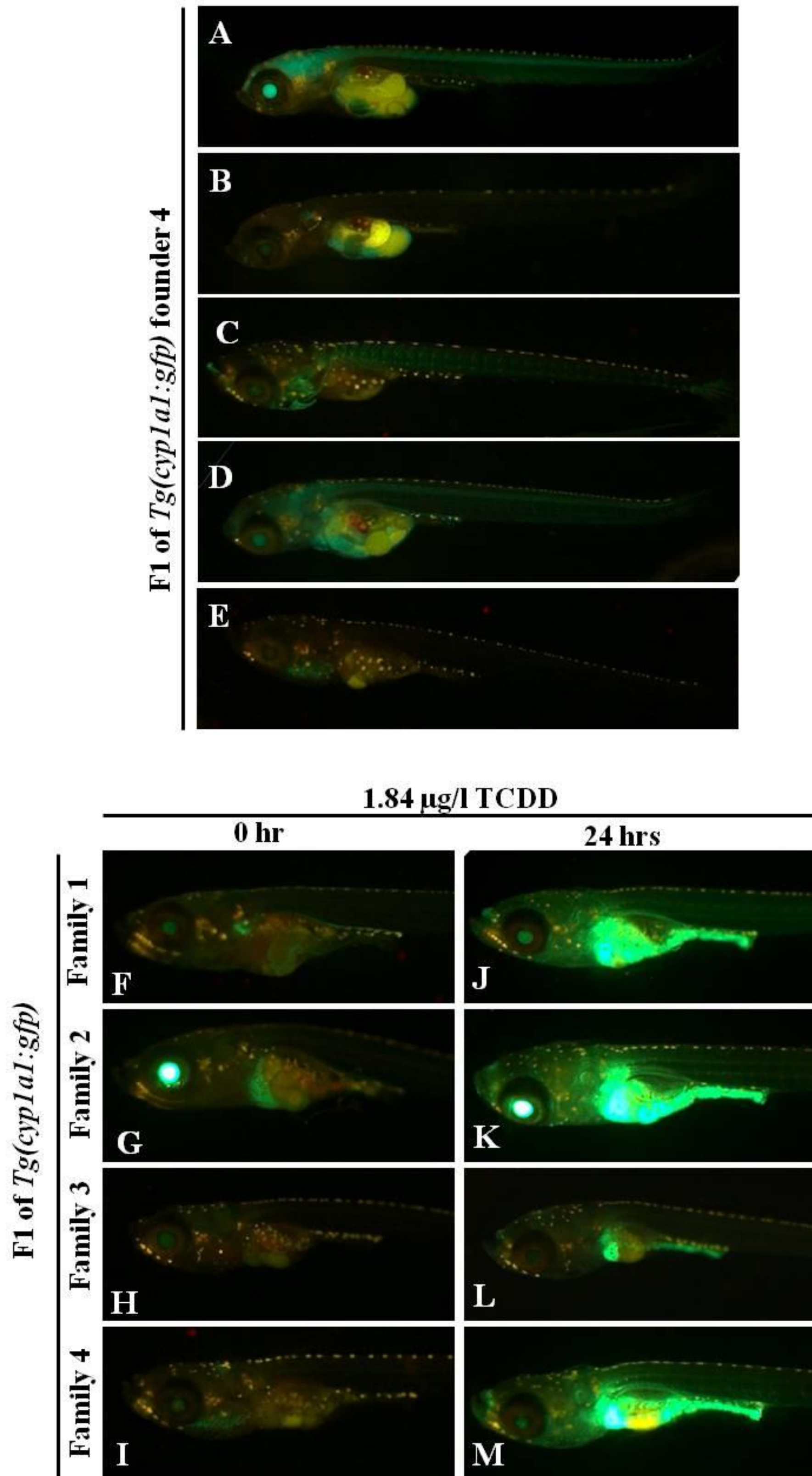

F1 of Tg(cyp1a1:gfp) founder 4
A
B
C
D
E
1.84 µg/l TCDD
0 hr
24 hrs
F1 of Tg(cyp1a1:gfp)
Family 1
Family 2
Family 3
Family 4
F
G
H
I
J
K
L
M

Figure 3.12 GFP expression in F1 embryos of Tg(*cyp1a:gfp*). (A-E) Variables patterns of constitutive GFP expressions in F1 progenies of Tg(*cyp1a:gfp*) founder 4. Images were taken from 1-3 dph fry and five distinct patterns are shown: (A) GFP expression in the notochord, brain, pectoral fins, olfactory pits and lens; (B) GFP expression in the otic vesicle, yolk and lens; (C) GFP expression in vertebra, lens, yolks, lips and weakly in muscle; (D) GFP expression in the notochord, brain, hearts, livers, gut, lens, gills and weakly in muscle; and (E) GFP expression in gills and some epithelial skin cells around abdomen and sometimes in lens. (F-M) TCDD-induced GFP expression of F1 *Tg(cyp1a1:gfp)* fry from all of the four transgenic founder families are shown. Images were taken from 3-7 dph fry. The same fry were shown before (F-I) and after 24 hrs of TCDD exposure (J-M). Note that in spite of variable constitutive and weak GFP expression before TCDD exposure, all of *Tg(cyp1a:gfp)* fry showed strong GFP expression in the liver, intestine and kidney. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder.

3.2.4 Multiple insertions in founder's family

It had been demonstrated in zebrafish that Ac transposase introduced multiple insertions in the genome and the average number of insertions in F0 founders was 4 (Emelyanov *et al.*, 2006). In the present study, 6 out of 14 positive transgenic founders (43%) had a germline transmission rate of more than 50% to their F1 progeny (Table 3.1), which was significantly higher than the expected Mendelian ratio based on crossing between heterozygote and a wild type. Furthermore, we also noticed that several distinct patterns of GFP expression in F1 Tg(*cyp1a:gfp*) fry from the same transgenic founder presumably due to chromosomal effect from different insertion loci (Fig 3.12). Thus, it was likely that at least some founders had multiple transgene insertions.

To verify occurrence of multiple insertions, Southern blot analysis was carried out to determine transgene insertions in genomic DNAs. Genomic DNAs were double-digested with restriction enzymes-MfeI and NdeI, where MfeI cut the transgene once and NdeI was to further digest the medaka genome without cutting the transgene. Neither MfeI nor NdeI cut the region of the two hybridization probes, promoter and *gfp* probes for each of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) (Fig. 3.13A).

Southern blot analysis was performed on Tg(*hsp70:gfp*) founder 1 family which had produced 100% transgenic F1 progeny (Table 3.1) by using the *hsp70* promoter probe and *gfp* probe. As shown in Fig. 3.13B and C, multiple bands were detected by both *hsp70* promoter and *gfp* probes. By comparison to wild type control (Fig. 3.13B, lane 1), in the pooled transgenic fry sample (Fig 3.13B, lane 2), three (#1,3,5) out of seven hybridized bands were endogenous *hsp70* fragments and the other four (#2,4,6,7) were likely from transgenes. This result was further confirmed by *gfp* probe hybridization of the same blot (Fig. 3.13C, lane 2) where the extra four

bands (#2,4,6,7) were hybridized. In the four individual F1 adult DNA samples (Fig 3.13B lane 3-6), while all of them contain the three endogenous *hsp70* fragments (#1,3,5), a variation in inheritance of transgenes was observed. Again, this was further confirmed by the *gfp* probe hybridization (Fig 3.13C, lane 3-6).

Similarly, Southern blot was performed on the F3 generation and its F2 transgenic parent of Tg(*cyp1a:gfp*) founder 1 family, where its descendants have shown at least two GFP phenotypes (data not shown). Southern blot was probed with the *cyp1a* promoter probe and *gfp* probe (Fig. 3.13A). In *cyp1a* probe hybridization, one band (#2) is detected in wild type control (Fig. 3.13D, lane 1) but two additional bands (#1,3) were detected in the F1 parent (Fig 3.13D, lane 2). Probing the same blot with the *gfp* probe confirmed the bands (#1,3) were from transgenes (Fig 3.13E, lane 2). The individual F2 offsprings (Fig 3.13D Lane 3-6) have combinations of inheritance of transgenes (#1 only, #1 and #3 or #3 only) which was further confirmed by the *gfp* probe (Fig 3.13E, Lane3-6).

All the above analysis from both Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) families indicated that there were multiple insertions of transgene in the genome.

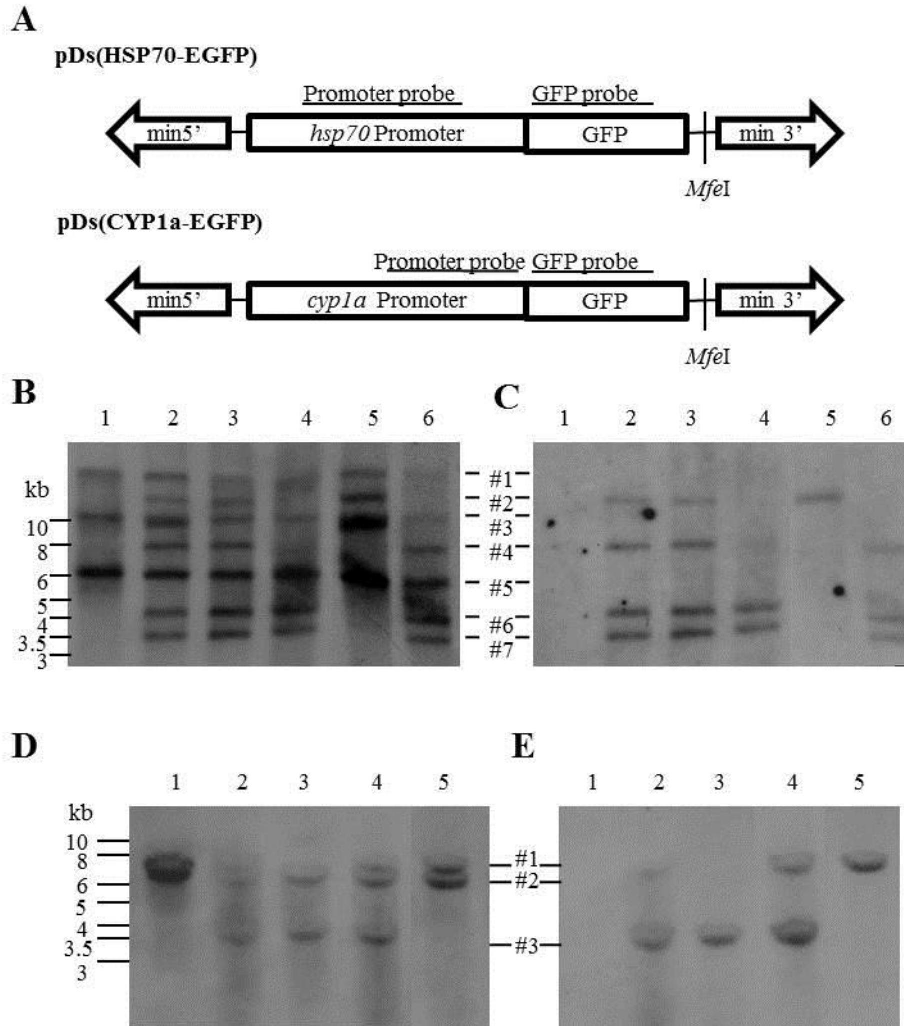


Figure 3.13 Analysis of transgene insertion. (A) DNA constructs for generation of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*). The promoter probes and *gfp* probes used in southern hybridization are indicated, together with a restriction enzyme site, MfeI, and minDs elements. (B-E) Southern blot analysis of transgene insertion. Molecular weights are indicated on the left. In (B,C), the same blot of Tg(*hsp70:gfp*) founder 1 family was probed with the *hsp70* promoter probe (B) and subsequently stripped and rehybridized with the *gfp* probe (C). Lane 1, wild type medaka; lane 2, 40 pooled 1-2 dph F1 transgenic fry to represent combined insertions sites in the germ cells of the transgenic founder; lane 3-6, F1 individual adult offspring. In (D, E), the same blot of Tg(*cyp1a:gfp*) F1 family was probed with *cyp1a* promoter probe (D) and subsequently stripped and rehybridized with *gfp* probe (E). Lane 1, wild type medaka; lane 2, F1 parent; lane 3-5, individual F2 adult offspring.

3.3 Selection of F1 for establishing biomonitoring lines

For the purpose of establishing biomonitoring transgenic line, F1 individuals were first screened for low constitutive GFP background and tested for GFP expression induction by relevant inducers to confirm their inducibility. Low constitutive GFP background of transgenic fish was preferred because it was easier to detect an increase in GFP signal during induction and to reduce false positive signal. We also wished to select transgenic lines which had only a single transgene insertion for characterization so that their future generations retained the same features in uniform genetic background.

3.3.1 Determination of transgene insertion number by Mendelian inheritance

F2 embryos of the low GFP constitutive background F1 fish were screened for transgenic positive embryos and the statistics was summarized in Table 3.2. Some of the F1 fish produced about 50% transgenic F2, which was consistent with the prediction of a single transgenic insertion based on Mendelian genetic law. Thus those F1 fish likely had single transgenic insertion and were further characterized. These include two F1 fish from Tg(*hsp70:gfp*) founder 1 family, designated as Tg(*hsp70:gfp*) 1.1 and Tg(*hsp70:gfp*) 1.2, and three F1 fish with each from Tg(*cyp1a:gfp*) founder 1, founder 3 and founder 4 families namely Tg(*cyp1a:gfp*) 1.1, 3.2 and 4.2.

Table 3.2 Transgene inheritance ratio of selected transgenic F1 individual

Transgenic F1	No. of transgenic embryos	No. of non transgenic embryos	Percentage of transgenic inheritance
Tg(*hsp70:gfp*) 1.1	116	124	48.3*
Tg(*hsp70:gfp*) 1.2	103	95	52.0*
Tg(*hsp70:gfp*) 1.3	27	15	64.3
Tg(*cyp1a:gfp*) 1.1	54	49	52.4*
Tg(*cyp1a:gfp)* 1.2	101	48	67.7
Tg(*cyp1a:gfp*) 3.2	109	105	50.9*
Tg(*cyp1a:gfp*) 4.2	92	93	49.3*

* Ratio fit Mendelian ratio of heterozygous single transgene outcross with chi-square test, sig of 0.05.

3.3.2 Confirmations of single insertion by Southern blot hybridization.

To demonstrate a single copy of transgenic insertions, Southern blot of wild type and Tg(*hsp70:gfp*) 1.1 and 1.2 were first hybridized with *hsp70* promoter probe (Fig. 3.14A) and later with *gfp* probe (Fig. 3.14B). Three fragments (#1,#2,#4) were detected in *hsp70* promoter probe hybridization of wild type sample (Fig. 3.14A, lane 1). There was an additional band (#3) in both Tg(*hsp70:gfp*) 1.1 and 1.2 (Fig. 3.14A, lane 2 and 3) other than the three fragments (#1,#2, #4) at the similar positions. This band (#3) was confirmed to be transgene upon hybridization by the *gfp* probe (Fig. 3.14B, lane 2 and 3). Thus, both genomes of Tg(*hsp70:gfp*) 1.1 and 1.2 contained only a single copy of transgene and most likely their genomic compositions were the same as both lines could inherit the same transgenic insertion.

Genomic DNA from Tg(*cyp1a:gfp*) 1.1, 3.2 and 4.2 were also used for Southern blot hybridization firstly by the *cyp1a* promoter probe (Fig. 3.14C), followed by the *gfp* probe (Fig. 3.14D). Only one band (#1) was detected in wild type fish (Fig. 3.14C, lane 1) with the *cyp1a* promoter probe. Tg(*cyp1a:gfp*) 3.2 and Tg(*cyp1a:gfp*) 4.2 each contained one additional band (Fig. 3.14C, #2 in lane 3 and #3 in lane 4) that was detected by *gfp* probe hybridization (Fig. 3.14D, lane 3 and 4). Although only one intense band (#1) was detected in the *cyp1a* promoter probe hybridization (Fig. 3.14C, lane 2) for Tg(cyp1a1:gfp) 1.1, *gfp* hybridization (Fig. 3.14C, lane 2) detected the same band position (#1). Thus, it is likely that the transgenic fragment was overlapped with the endogenous *cyp1a* promoter fragment because of their similar sizes. As such, more intense band (#1) (Fig 3.14C, lane 2) was observed with the *cyp1a* promoter probe hybridization. Thus, Tg(*cyp1a:gfp*) 1.1, 3.2 and 4.2 all contained a single copy of transgene at different insertion loci.

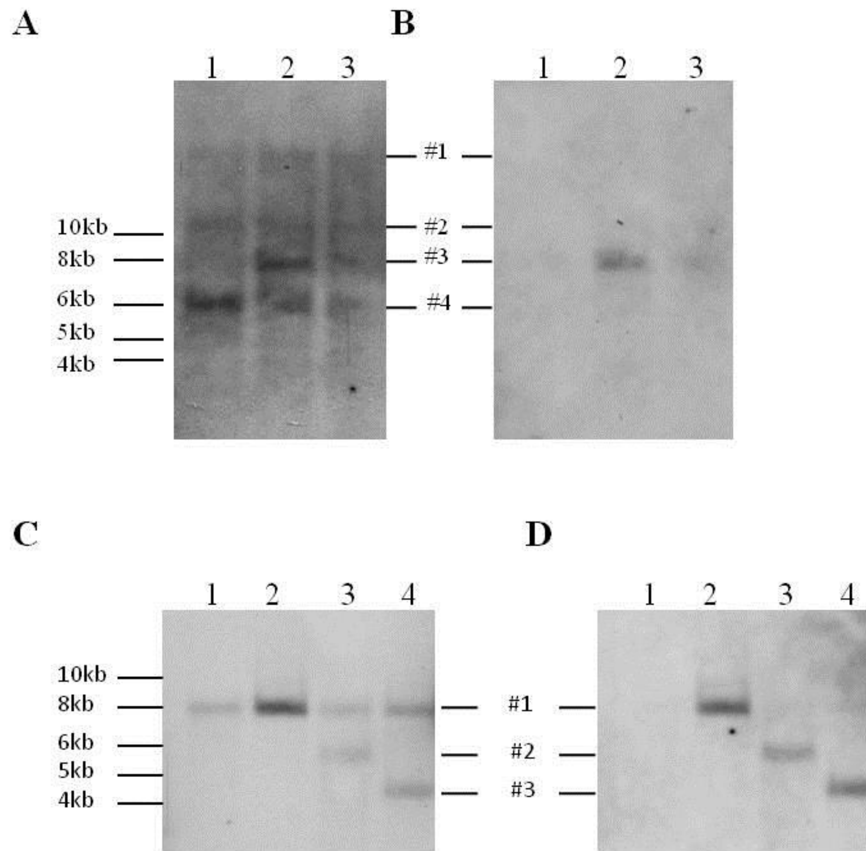


Figure 3.14 Demonstration of single insertion in selected transgenic lines by Southern blot hybridization. (A,B) Analysis of genomic DNAs from Tg(*hsp70:gfp*) 1.1 and 1.2.The same Southern blot of selected Tg(*hsp70:gfp*) F1 was probed with *hsp70* promoter probe (A) and stripped and rehybridized with *gfp* probe (B). Lane 1, wild type medaka; lane 2, Tg(*hsp70:gfp*) 1.1 ; lane 3,Tg(*hsp70:gfp*) 1.2. (C,D) Analysis of genomic DNAs from Tg(*cyp1a:gfp*) 1.1, 3.2 and 4.2. The same southern blot of selected Tg(*cyp1a:gfp*) F1 was probed with *cyp1a* promoter probe (C) and stripped to reprobe with *gfp* probe (D). Lane 1, wild type medaka; lane 2, Tg(*cyp1a:gfp*) 1.1; lane 3, Tg(*cyp1a:gfp*) 3.2; lane 4, Tg(*cyp1a:gfp*) 4.2. Molecular weights are indicated on the left.

3.3.3 Analysis of flanking sequences

Using LM-PCR approach, the genomic flanking sequences of transgenic lines were cloned and sequenced as shown in Fig. 3.15. The flanking sequences of both Tg(*hsp70:gfp*) 1.1 and 1.2 were identical, confirming that they have the same transgene insertion. Insertions of hAT transposon superfamily members, which include Ac/Ds, generate 8-bp target site duplications that flank the transposon insertion (Weil & Kunze, 2000). Indeed, the 8-bp target site duplications were identified beside the minDs element for Tg(*hsp70:gfp*) 1.1, 1.2 and the three Tg(*cyp1a:gfp*) lines (Fig. 3.15, bold underlined letters). However, in Tg(*cyp1a:gfp*) 4.2, there was an additional short sequence (GAGGAG, red bold underlined letters), which seem to be complementary to the 8-bp target site duplication sequence immediately upstream.

BLAST search of the flanking sequences was performed for the genomic integration site. The derived flanking sequence (Fig 3.16A) of Tg(*hsp70:gfp*) 1.1 was located in medaka contig scaffold 797, with 100% identity (Fig 3.16B). The integration site happened in the intronic regions of two novel putative protein coding genes, ENSORLT00000023316 (positive direction) and ENSORLT00000023310 (negative direction) (Fig. 3.16C). The integration site of Tg(*cyp1a:gfp*) 1.1 was located in medaka contig scaffold 3334, with 93.6% sequence identity (Fig. 3.17B), based on almost whole length sequence of the flanking region (Fig. 3.17A). No putative gene was identified within 2.5 kb of both upstream and downstream sequences, of the insertion site (Fig. 3.17C). For Tg(*cyp1a:gfp*) 3.2, all of the flanking sequence (Fig. 3.18A) was located in chromosome 3 with 100% sequence identity (Fig. 3.18B). No putative gene was located within 2.5 kb region upstream or downstream of the insertion site (Fig. 3.18C). Lastly, only about 70% (51/73) of the

flanking sequence (Fig. 3.19A) of Tg(*cyp1a:gfp*) 4.2 was located in chromosome 3 with 94.3% identity and E value of 4.2e-13 (Fig. 3.19B). The 20 bp upstream of the flanking sequence was not similar to that of genomic sequence of the BLAST location. Also, no putative gene was located within 2.5 kb region upstream and downstream of the insertion site (Fig. 3.18C).

It is interesting to note that the derived flanking sequences of some insertions were not 100% identical to that of genomic database, perhaps due to single nucleotide polymorphism or individual genome variation. Furthermore, insertion sites of Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) 1.1 were identified on contig regions which were not mapped to the chromosome yet. Nevertheless, based on BLAST results, only Tg(*hsp70:gfp)* was found in intronic region of two putative annotated genes, however no abnormality was discovered in Tg(*hsp70:gfp*) , suggesting that the insertion of transgene may not affect the function of either gene.

Tg(*hsp70:gfp*) 1.1 & 1.2

….GGACCGAGTG**GTTTTAAG***cagg(min5'Ds)*-----*(min3'Ds)cccta***GTTTTAAG**TAAATCCTTC…

Tg(*cyp1a:gfp*) 1.1

...TTGCCAAGGC**CCCTTACC***cagg(min5'Ds)*----*(min3'Ds)cccta***CCCTTACC**CCCCACCAGG…

Tg(cyp1a:gfp) 3.2

…TCCAAATTGT**ATTAAATC***cagg(min5'Ds)*----*(min3'Ds)cccta***ATTAAATC**CAGTGTGCTG….

Tg(*cyp1a:gfp*) 4.2

…TTTCACGTCC**TCTCCTC****AGAGGAGTA***cagg(min5'Ds)*----*(min3'Ds)cccta***TCTCCTCT**CTCCCTACTG…

Figure 3.15 Flanking sequence of transgene integration. Only sequences flanking the Ds element of the transgenes are shown. Ds end sequences are shown in italic lower case type; flanking genomic sequences are shown in uppercase types. The classical 8-bp direct target site duplication is shown in bold and underlined. Bold and red sequence in Tg(*cyp1a:gfp*) 4.2 was suspected to be a result of aberrant target site duplication.

A

5'CACAACCAGAACCTTGGACCGGTCCGGTGGACCGAGTGGTTTTAAGTAAATCCTTC
AGCCTTTAGCCCAAGTGTTCTCAATCTGCTTACGCTTCCTGAGAAGCCGTG 3'

B

```
Query location      : hsp70:gfp 1.1        1 to   107 (-)
Database location   : scaffold797 75079 to 75185 (+)
Genomic location    : scaffold797 75079 to 75185 (+)
Alignment score     : 107
E-value             : 2.4e-55
Alignment length    : 107
Percentage identity: 100.00

Query:    107 CACGGCTTCTCAGGAAGCGTAAGCAGATTGAGAACACTTGGGCTAAAGGCTGAAGGATTT 48
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 75079 CACGGCTTCTCAGGAAGCGTAAGCAGATTGAGAACACTTGGGCTAAAGGCTGAAGGATTT 75138

Query:     47 ACTTAAAACCACTCGGTCCACCGGACCGGTCCAAGGTTCTGGTTGTG 1
              |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 75139 ACTTAAAACCACTCGGTCCACCGGACCGGTCCAAGGTTCTGGTTGTG 75185
```

C

Figure 3.16 Identification of transgenic insertion site in Tg(*hsp70:gfp*) 1.1. (A) Derived flanking sequences where red letters represent the 8-bp target duplication and the underlined represent the query sequence used for BLAST search. (B) BLAST results against medaka genome sequence from Ensembl. (C) Contig view of the query sequence in the medaka genome. Red bar represent the query sequence position. Note that exon and intron region of the Ensembl gene are represented by red box and dotted red line respectively on the Ensembl gene track.

A

5'ATGGCCCGACCAGGCTCAGGCCACAGTTGGAAATTTGGCGGGGCACAGCGCTCAGGGG
CAAGGGCCAGGAACCACCCCCCAGGGACACGAACACCCCCGGCTCAGGTGTATTGTGAA
CCCCCTCCCCGCAAGGAGAGAGCGCCTCCGGGCCCAGGAAACCGGCACCCAAGGGACAC
GGCCGCTGTTGCCAAGGCCCCTTACCCCCCACCAGGGAAGGGGTAGGGGACAGATGGTC
CTAGGTCCCACCTTCCTTGCAAAATGTGTGTGCGTGTGTTTGAGAGGATGTGTGTGTGCAT
GCAT3'

B

```
Query location      : cyp1a:gfp 1.1 4 to  297 (-)
Database location   : scaffold3334 1414 to 1707 (+)
Genomic location    : scaffold3334 1414 to 1707 (+)
Alignment score     : 221
E-value             : 1.3e-121
Alignment length    : 297
Percentage identity: 93.60
Query:   297 CATGCACACACACATCCTCTCAAACACACGCACACACATTTTGCAAGGAAGGTGGGACCT 238
             |||||||||||||| ||||||||| ||| ||||||||||||||||||| |||||||||||
Sbjct:  1414 CATGCACACACACACCCTCTCAAAGACATGCACACACATTTTGCAAGGTAGGTGGGACCT 1473

Query:   237 AGGACCATCTGTCCCCTACCCCTTCCCTGGTGGGGGTAAGGGGCC-TTGGCAACAGCGG 179
             |||||||||||||||||| ||||||||||||||||||| |||||| |||||||||||||
Sbjct:  1474 AGGACCATCTGTCCCCTAGCCCTTCCCTGGTGGGGGTACGGGGCCCTTGGCAACAGCGG 1533

Query:   178 CCGTGTCCCTTGGGTGCCGGTTTCCTGGCCCGGAGGCGCTCTCTCCTTGCGGGGAGGGG 119
             |||||||||||||||||||||||||||||||||||| ||| ||||| |||||||||||||
Sbjct:  1534 CCGTGTCCCTTGGGTGCCGGTTTCCTGGCCCGGAGGTGCTTTCTCCGTGCGGGGAGGGG 1593

Query:   118 GTTCACAAT-ACACCTGAGCCGGGGGTGTTCGTGTCCCTGGGGGGTGG-TTCCTGGCCCT 61
             ||||||| | ||||||||||||||||||| ||||||| |||||||||| ||| ||| |||
Sbjct:  1594 GTTCACA-TGACACCTGAGCCGGGGGTGTCCGTGTCCGTGGGGGGTGGGTTC-TGGTCCT 1651

Query:    60 TGCCCCTGAGCGCTGTGCCCCGCCAAATTTCCAACTGTGGCCTGAGCCTGGTCGGGC 4
             ||||||||||||||| |||||||||||||||||||||||||| ||||||||||||||
Sbjct:  1652 TGCCCCTGAGCGCTGGGCCCCGCCAAATTTCCAACTGTGGCC-GAGCCTGGTCGGGC 1707
```

C

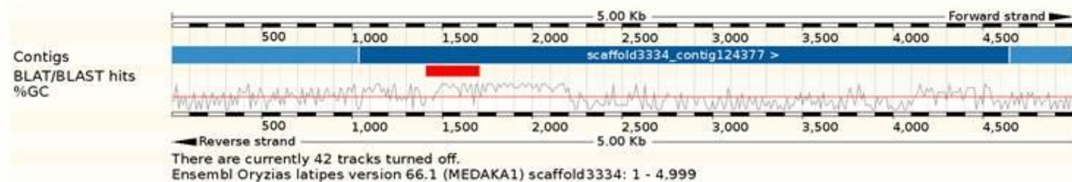


Figure 3.17 Identification of transgenic insertion site in Tg(*cyp1a:gfp*) 1.1. (A) Derived flanking sequences where red letters represent the 8-bp target duplication and the underlined represent the query sequence used for BLAST search. (B) BLAST results against medaka genome sequence from Ensembl. (C) Contig view of the query sequence in the medaka genome. Red bar represent the query sequence position.

A

5' ATGTGTCCAAATTGTATTAAATCCAGTGTGCTGGATAGTACAGCAATATCAT 3'

B

```
Query location     : cyp1a:gfp 3.2        1 to       50 (+)
Database location  : 3       30719452 to 30719501 (+)
Genomic location   : 3       30719452 to 30719501 (+)
Alignment score    : 50
E-value            : 2.2e-21
Alignment length   : 50
Percentage identity: 100.00
Query:         1 ATGTGTCCAAATTGTATTAAATCCAGTGTGCTGGATAGTACAGCAATATC 50
                 ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 30719452 ATGTGTCCAAATTGTATTAAATCCAGTGTGCTGGATAGTACAGCAATATC 30719501
```

C

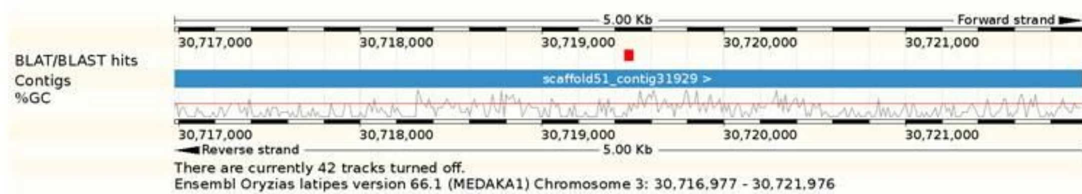


Figure 3.18 Identification of transgenic insertion site in Tg(*cyp1a:gfp*) 3.2. (A) Derived flanking sequences where red letters represent the 8-bp target duplication and the underlined represent the query sequence used for BLAST search. (B) BLAST results against medaka genome sequence from Ensembl. (C) Contig view of the query sequence in the medaka genome. Red bar represent the query sequence position.

A

5'ATGTGCTGTGACTAAGATTCGGGGGGGGGGGTCTTCTTTCACGTCCTCTCCTCTCTCCCT
ACTGTCTCATGCAT3'

B

```
Query location     : cyp1a:gfp 4.2       21 to       71 (+)
Database location  : 3       22611216 to 22611268 (+)
Genomic location   : 3       22611216 to 22611268 (+)
Alignment score    : 41
E-value            : 4.2e-13
Alignment length   : 53
Percentage identity: 94.34
Query:       21 GGGGGGGGGGGTCTTCTTTCACGTCCTCTCCTC-CTCCCTACTGT-CTCATGC 71
                ||||||||||||||||||||||||||||||||| |||| |||||| |||||||
Sbjct: 22611216 GGGGGGGGGGGTCTTCTTTCACGTCCTCTCCTCTCTCCGTACTGTTCTCATGC 22611268
```

C

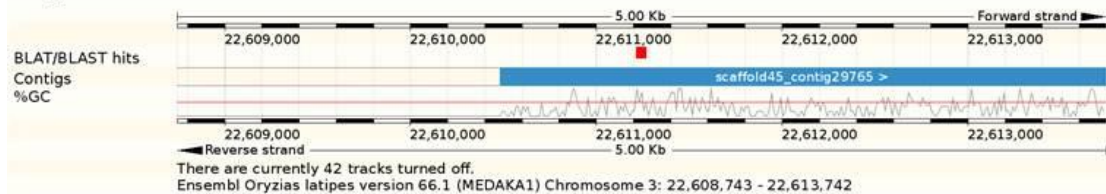


Figure 3.19 Identification of transgenic insertion site in Tg(*cyp1a:gfp*) 4.2. (A) Derived flanking sequences where red letters represent the 8-bp target duplication and the underlined represent the query sequence used for BLAST search. (B) BLAST results against medaka genome sequence from Ensembl. (C) Contig view of the query sequence in the medaka genome. Red bar represent the query sequence position.

3.3.4 Description and selection of best transgenic lines for biomonitoring purpose

3.3.4.1 Tg(*hsp70:gfp)* line

Based on Southern blot (Fig. 3.14A, B) and flanking sequences (Fig 3.15), both Tg(*hsp70:gfp*) 1.1 and 1.2 likely had the same inheritance transgenic insertion inherited from the same F0 founder parent. Therefore Tg(*hsp70:gfp*) 1.1 was used for future characterization.

As mentioned in Section 3.2.3, constitutive GFP expression was observed in the lens of Tg(*hsp70:gfp*) 1.1 embryos from 3 dpf onwards and the lens expression remained throughout their life cycle. No other constitutive GFP expression was observed in the fish except that when the female fish has reached maturity, the ovum in its ovary expressed GFP (Fig. 3.20A). The ovum GFP expression was persistent as maternal GFP expression existed ubiquitously in both embryonic cells and yolk sac in early development (Fig. 3.20B-D) but diminished gradually as the developing embryo reached 6 dpf. In contrast, this constitutive GFP expression was not observed in transgenic embryos derived from male transgenic fish (data not shown).

The F2 embryos of Tg*(hsp70:gfp)* 1.1 after crossing with a wild type fish, with 50% of the embryos expected to be transgenic, was subjected to heat shock treatment at 37 ^{o}C at 2 dpf where lens specific GFP transgenic marker were not developed yet (Fig. 3.21A, E). Six out of nine embryos showed GFP expression after heat shock treatment (Fig. 3.21F) however no GFP expression was induced in the control group (Fig. 3.21B). GFP expression was observed ubiquitously in the embryonic body with the strongest intensity observed in the head region (Fig. 3.21G). This was consistent with the in situ hybridization result where *hsp70* mRNA was expressed ubiquitously in the heat shocked embryo (Fig. 3.21H) and no *hsp70* transcript was detected in the

non-heat shock control (Fig. 3.21D), thus demonstrating that the transgene expression is similar to that of *hsp70*.

The F2 outcross embryos of Tg(*hsp70:gfp*) 1.1 were also subjected to mercury treatment from 6 hpf. After 72 hours of treatment, except for lens-specific GFP expression in three transgenic embryos (Fig. 3.22C), no GFP expression was observed in the control group of six embryos (Fig. 3.22B). In the group treated with 50 µg/l mercury chloride, weak GFP expression (Fig. 3.22F) was observed in the embryonic body of only two transgenic embryos (Fig. 3.22E), which were identified by their constitutive lens-specific GFP expression. At 100 µg/l mercury chloride, stronger expression was observed in embryonic body and yolk sac (Fig. 3.22I) in all four transgenic embryos (Fig. 3.22H). Thus, there is a dose-dependent induction of GFP expression towards mercury chloride in all transgenic Tg(*hsp70:gfp*) embryos.

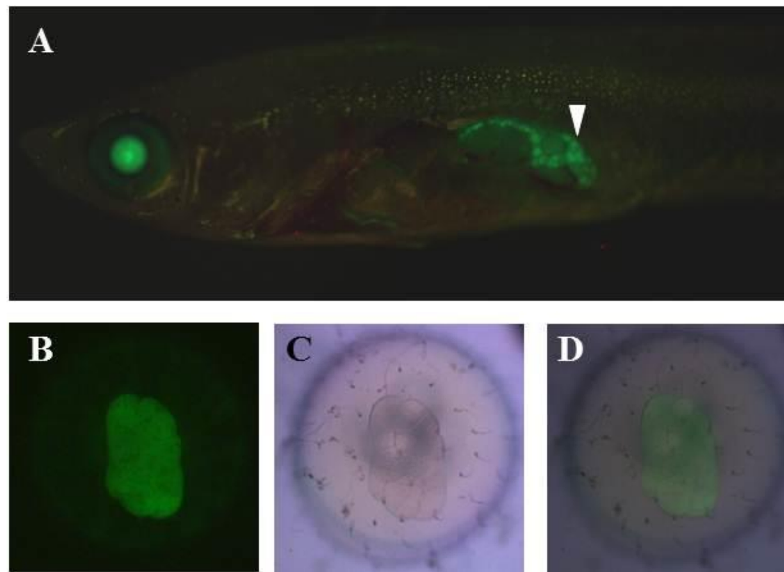


Figure 3.20 Constitutive GFP expression of Tg(*hsp70:gfp*) 1.1. (A) Abdomen dissection of a 6-month old female fish under fluorescent view. A white arrowhead indicates the position of ovary. Note that there was constitutive GFP expression in the lens of transgenic fish. (B-D) Maternal GFP expression in which fluorescent (B), brightfield (C) and merged (D) image was taken of animal pole of 16-cell stage blastula.

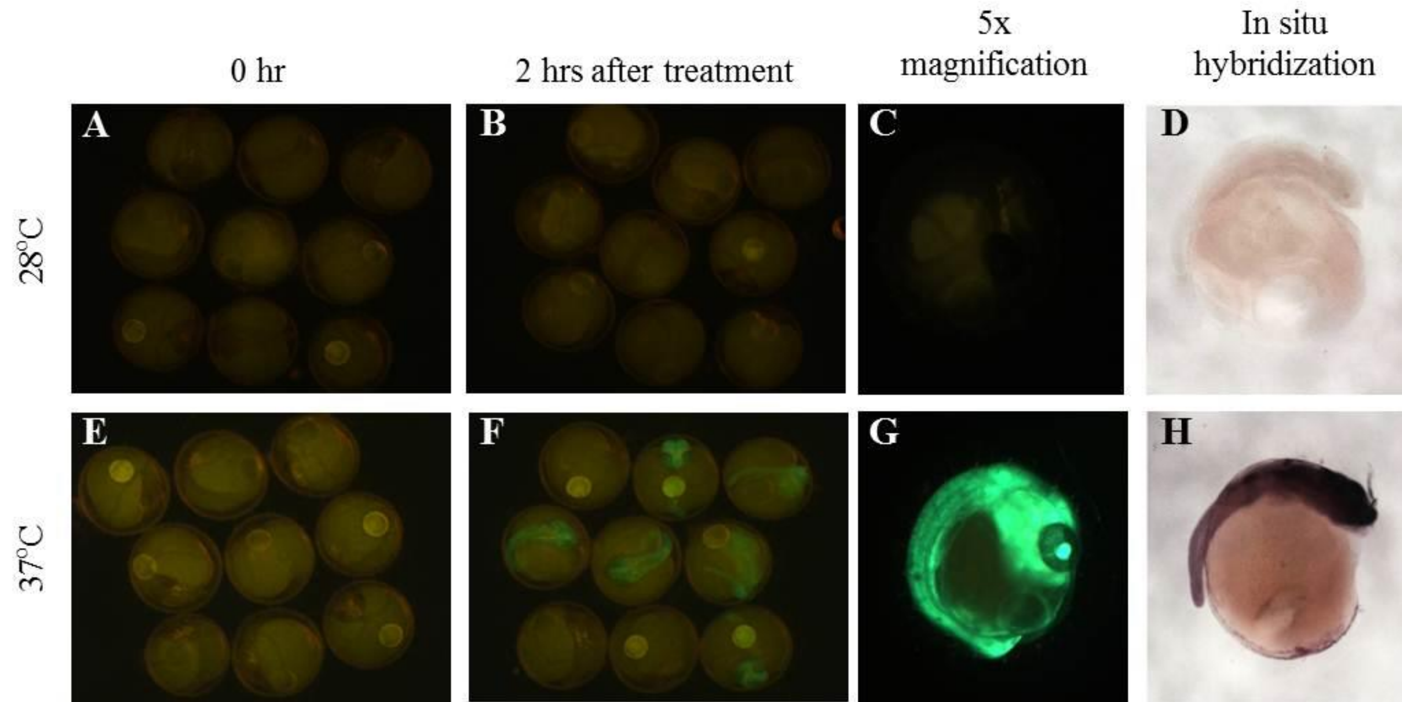


Figure 3.21 Heat shock induced GFP expression in Tg(*hsp70:gfp*) 1.1 F1 embryos. 2-dpf embryos were heat shocked at 37 ^{o}C for 2 hours before returning back to 28 ^{o}C. The 2-dpf embryos in control were incubated at 28 ^{o}C instead. No GFP expression was observed in 2-dpf embryos prior to treatment (A, E). 2 hours after heat shock treatment, GFP expression was induced in some embryos (F) while those at 28°C (B) were not observed to express GFP. Note that not all of the embryos were transgenic as it was outcross of Tg(*hsp70:gfp*) 1.1 and wild type. (C) and (G) is the higher magnification of the representative trangenic embryo in (B) and (F) respectively. (D, H) In situ hybridization of 2 dpf wild type with *hsp70* antisense probe under 28°C (D) or heat shock treatment, 37°C (H).

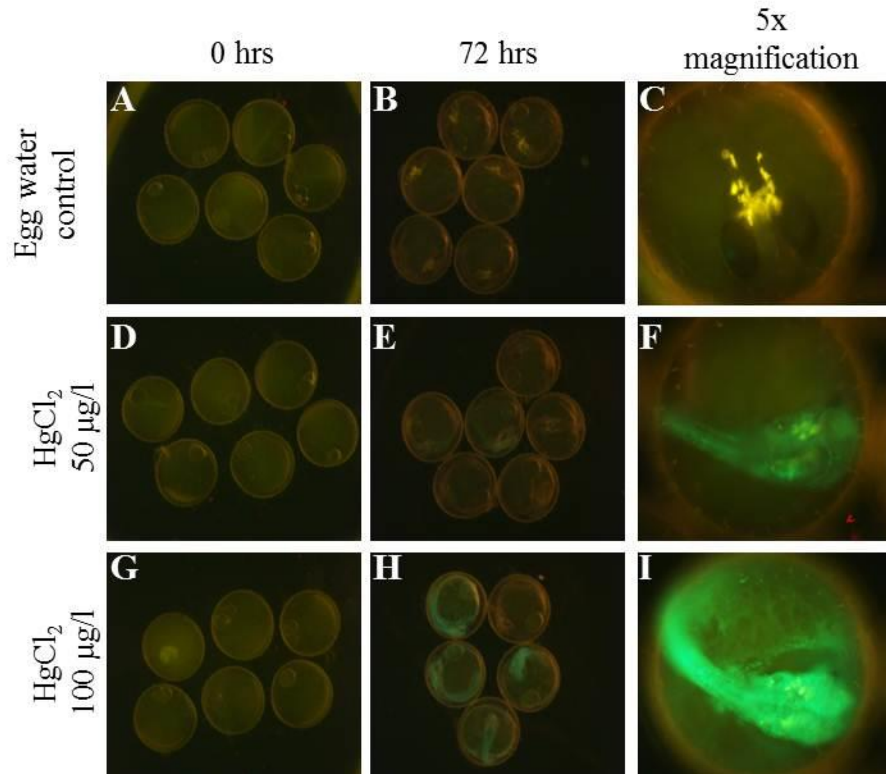


Figure 3.22 Dosage-dependent induction of GFP expression by $HgCl_2$ in F1 embryos of Tg(*hsp70:gfp*) 1.1. Pictures of the same batch of embryos were taken before (A,D,G) and 72 hrs after (B,E,H) exposure. (C,F,I) 5x magnification of dorsal view of a representative transgenic embryo from each group (B,E,H) respectively.

3.3.4.2 Tg(*cyp1a:gfp*) lines

Three lines of Tg(*cyp1a:gfp*) have different genomic loci of transgene insertions based on Southern blot analysis (Fig. 3.14C, D) and derived flanking sequences (Fig. 3.15). Often, different genomic loci influence the transgene expression, possibly by nearby transcriptional modulators in many transgenic fish studies (Grabher & Wittbrodt, 2008; Rocha *et al.*, 2004; Gong & Hew, 1995). Hence, it was of interest to determine the most sensitive and robust line to characterize for future biomonitoring application.

Except for occasional weak GFP expression in the yolk sac around the period from 2 dpf to 3 dpf, no constitutive expression was observed for neither Tg(*cyp1a:gfp*) 1.1 nor 4.2 line. As for Tg(*cyp1a:gfp*) 3.2, strong GFP was expressed in the neural fold region at 1 dpf (Fig. 3.23A, B) and the expression gradually diminished to two spots in the mid brain region from 3 dpf thereafter and remained even after hatching (Fig. 3.23C).

Since there was no visible transgenic marker to differentiate transgenic embryos from non transgenic embryos in Tg(*cyp1a:gfp*) 1.1 and 4.2 lines, it was difficult to determine the number of transgenic embryos used in the chemical exposure. Nevertheless, the F2 embryos, with expected 50% transgenic embryos, of each Tg(*cyp1a:gfp*) line were treated with a range of concentration of TCDD to determine their sensitivity. After 24 hrs, exposure to 322.0 ng/l (1 nM) TCDD induced GFP expression in the yolk, olfactory pits, kidney, liver and gut of transgenic embryos for all three lines (Fig. 3.24E, F, K, L, Q, R). At 80.5 ng/l (0.25 nM) TCDD, faint GFP expression was induced in the liver in Tg(*cyp1a:gfp*) 4.2 (Fig. 3.24P) as compared to DMSO control (Fig. 3.24N). Although faint GFP expression was also observed in kidney tubules in 80.5 ng/l (0.25 nm) TCDD-treated Tg(*cyp1a:gfp*) 3.2

fry (Fig. 3.24J), the similar observation was noted in DMSO control (Fig. 3.24H) possibly due to leaky expression of transgene. These results suggested that Tg(*cyp1a:gfp*) 4.2 was the most sensitive line towards TCDD.

Since GFP in Tg(*cyp1a:gfp*) was driven by endogenous *cyp1a* promoter, we expected that the presence of GFP indicated up-regulation of *cyp1a* transcription. We observed GFP expression in organs such as liver, kidney, gut, gills, olfactory pits, undifferentiated blood vessels at tail fin, blood vessels along the trunk and mast cells along lateral line and around eyes, in TCDD treated 1-3 dph fry of Tg(*cyp1a:gfp*) 4.2 after 24 hours of exposure (Fig. 3.25C, F, I, L and Fig. 3.37A). Hence GFP expression observed in these organs would signify that *cyp1a* was also induced in these organs under TCDD treatment. To compare with the endogenous *cyp1a* expression pattern, we performed in situ hybridization with *cyp1a* cDNA probe. In situ hybridization of TCDD treated fry (Fig. 3.25B, E, H, K) demonstrate the positive signal for *cyp1a* transcript in head kidney, liver, gut, mast cells, gills and olfactory pits, therefore corresponding to the GFP signal observed (Fig. 3.25C, F, I, L). The DMSO control (Fig. 3.25A, D, G, J) did not have these *cyp1a* hybridization signal in these organs. However, *in situ* hybridization could not conclude the presence of *cyp1a* transcripts in blood vessels and tail.

Newly hatched F2 larvae of each transgenic line were also exposed to BAP. Compared to DMSO control representative (Fig. 3.26A-C), in all three lines, induced GFP expression was observed in the liver and weakly in kidney (Fig. 3.26D, E, G, H, J, K). In addition, the tail fin of Tg(*cyp1a:gfp*) 3.2 (Fig. 3.26I) and 4.2 (Fig. 3.26L) were also observed to express GFP weakly.

In summary, all three Tg(*cyp1a:gfp*) lines responded to inducers such as TCDD and BAP. Generally, the intensity and pattern of Tg(*cyp1a:gfp*) 3.2 and 4.2

were similar while Tg(*cyp1a:gfp*) 1.1 had the least number of organs to express GFP. However, it appeared that Tg(*cyp1a:gfp*) 4.2 is the most suitable line among the three lines towards TCDD because of its nil constitutive GFP expression. Thus, Tg(*cyp1a:gfp*) 4.2 was selected to be characterized further in chemical exposures.

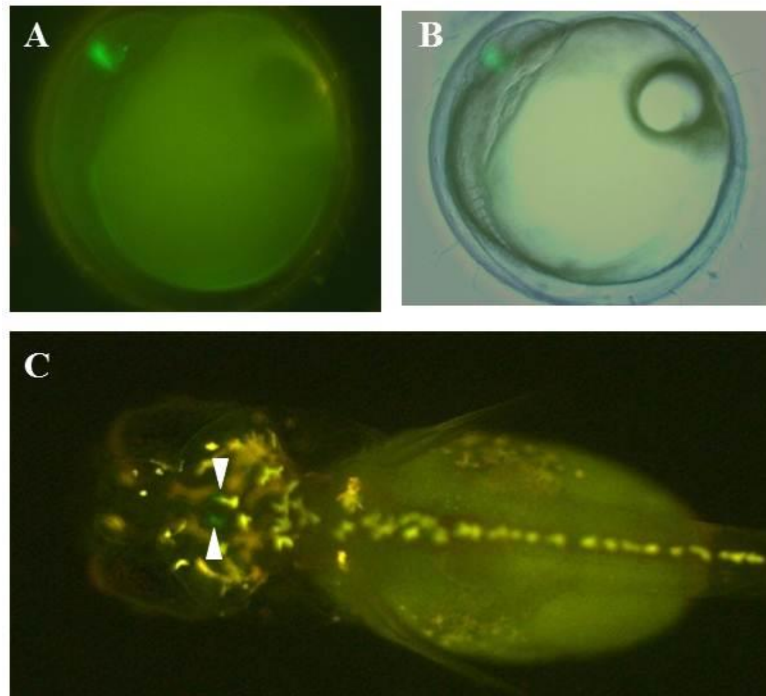


Figure 3.23 Constitutive GFP expression of Tg(*cyp1a:gfp*) 3.2. (A,B) GFP expression in the neural fold of 1 dpf embryo under fluorescent view (A) and merged view (B). (C) GFP expression was reduced to two spots in the mid brain of the newly hatched Tg(*cyp1a:gfp*) 3.2 larvae. White arrowheads point to the positions of GFP expression. Yellow regions observed were auto fluorescence of pigment cells and yolk.

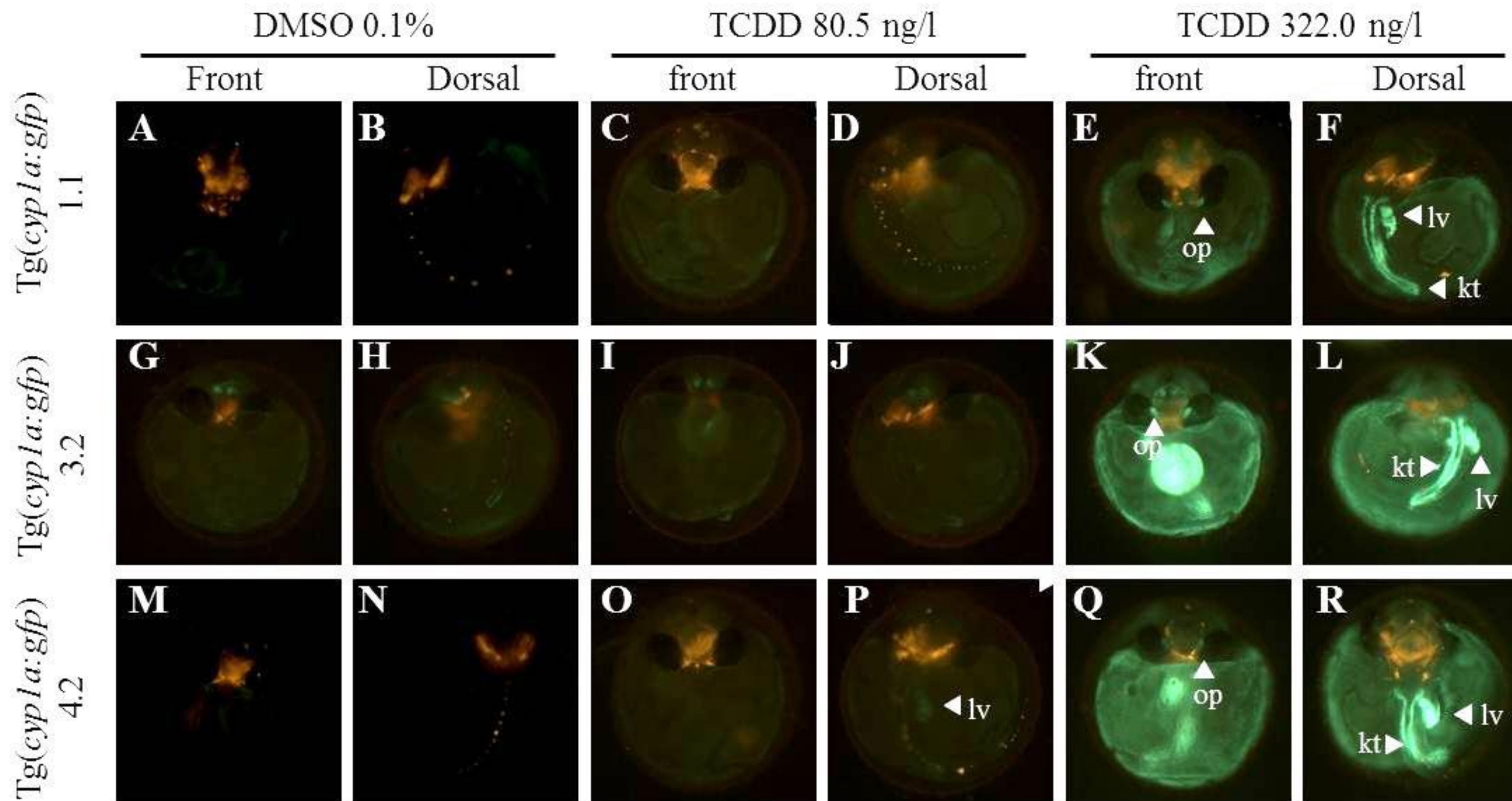


Figure 3.24 TCDD induction of GFP expression in selected Tg(*cyp1a:gfp*) lines. Representative images of GFP expression of transgenic embryo treated with range of TCDD concentration in Tg(*cyp1a:gfp*) 1.1 (A-F), Tg(*cyp1a:gfp*) 3.2 (G-L) and Tg(*cyp1a:gfp*) 4.2 (M-R). Front and dorsal views were taken of the same embryo.White head arrows indicate the positions of induced GFP expression. Abbreviations : kt, kidney tubules; lv, liver; op, olfacoty pit.

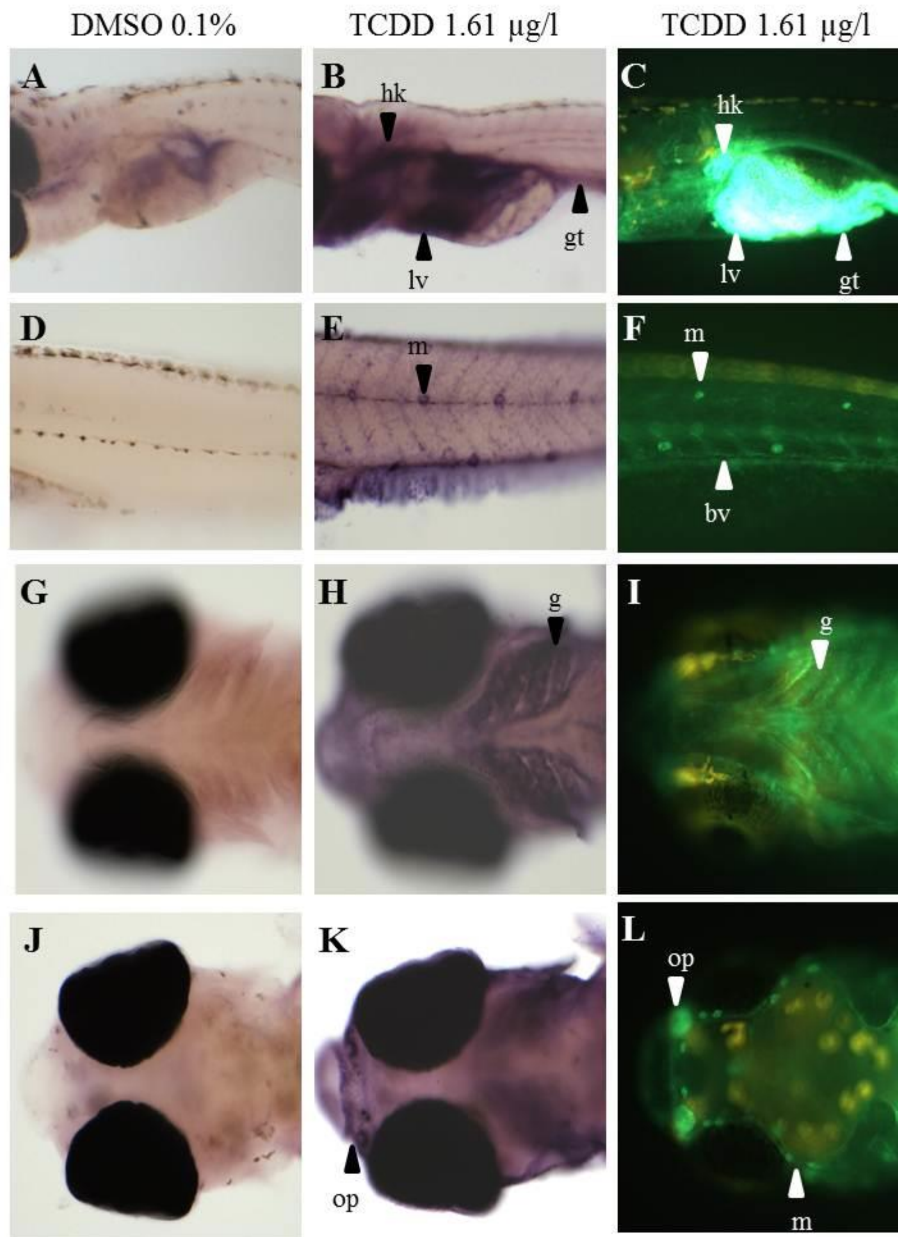


Figure 3.25 Corresponding endogenous expression of *cyp1a* gene to GFP expression in TCDD-treated fry. Whole mount in situ hybridization of *cyp1a* mRNA in vehicle control group, DMSO 0.1% (A, D, G, J) and 1.61 µg/l TCDD-treated wild type fry (B, E, H, K). GFP expression in 1.61 µg/l TCDD-treated transgenic fry(C, F, I, L). Abbreviations: o, olfactory pit; m, mast cells; g, gills; bv, blood vessel.

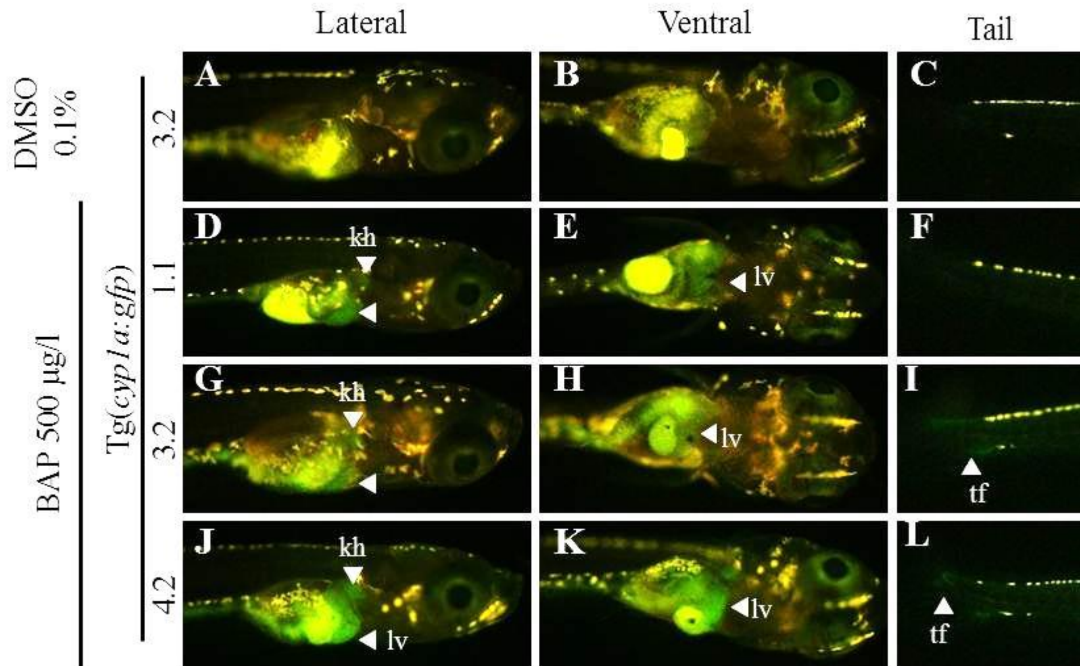


Figure 3.26 BAP induction of GFP expression in selected Tg(*cyp1a:gfp*) lines. (A-C) GFP expression in vehicle control (DMSO 0.1%) of Tg(*cyp1a:gfp*) 3.2. The images were representative of all transgenic lines treated with vehicle solvent. (D-L) Representative images of GFP expression of transgenic fry treated with BAP of Tg(*cyp1a:gfp*) 1.1 (D-F) , Tg(*cyp1a:gfp*) 3.2 (G-I) and Tg(*cyp1a:gfp*) 4.2 (J-L). White head arrows indicate the position of induced GFP expression. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder. Abbreviations: kh, kidney head; lv, liver; tf, tail fin.

3.4 Characterization of Tg(*hsp70:gfp*) for biomonitoring purpose

3.4.1 Heavy metals exposure of Tg(*hsp70:gfp*) fry

Since *hsp70* was observed to up-regulate in heavy metal exposure, three metal salt solutions, mercury chloride, cadmium chloride and sodium arsenate, were used for exposure of Tg(*hsp70:gfp*) fry (1-3 dph) to examine their responsiveness.

Preliminary 72 hours exposure of mercury chloride to1-3 dph wild type fry has shown that high mortality rate (87.5%) at 1000 µg/l. Exposure from 400 µg/l to 800 µg/l of mercury chloride showed mortality rate from 12.5% to 37.5%. No apparent deformities, abnormalities or mortality was observed in fry that were treated with concentrations at ≤ 200 µg/l of mercury chloride. Hence we treated the Tg(*hsp70:gfp*) 1-3 dph fry with mercury chloride from 200 µg/l to 1000 µg/l for 24 hours to determine GFP expression. At the lowest tested concentration (200 µg/l) of mercury chloride, GFP expression was observed weakly in the kidney and in some epithelial cells of the gills in 60% the fry (Fig. 3.27). Besides kidney and gills, GFP expression was also observed in other organs such as liver, skin and notochord (Fig. 3.27A) at concentrations higher than 200 µg/l. As quantified in Fig. 3.27B but disregarding the statistics in the 1000 µg/l group that had only 3 out of 15 fry survived, the number of fry that expressed GFP in kidney and liver increased with dosage. However, dosage-dependent effect was not observed in other organs such as gills, notochord and skin. The intensity of GFP expression, especially in liver, at 600 µg/l and above appeared to be strong.

Preliminary cadmium chloride exposure to 1-3 dph wild type fry had sudden high mortality rate (87.5%) observed at concentrations at 200 µg/l and above, while mortality rate from 37.5% to 62.5% was observed at concentrations from 25 µg/l to

100 μg/l, at 72 hours of exposure. Gross abdomen edema and curved spine were often observed in fry treated with ≥100 μg/l of cadmium chloride. There, the Tg(*hsp70:gfp*) fry (1-3 dph) were treated with cadmium chloride from 25 μg/l up to 400 μg/l for 24 hours to determine GFP expression. Kidney (Fig. 3.28A) seemed to be the most sensitive organs since the highest number of fry showed GFP fluroscence in the kidney at all the concentrations of cadmium chloride (Fig. 3.28B). Liver was the next sensitive organ. For example, at the lowest tested concentration (25 μg/l), about 40% of the fry expressed GFP in the kidney and 6.7% of the fry expressed GFP in the liver. At 50 μg/l, the number of fry expressing GFP in the kidney and liver increased to 75% and 41.7%, respectively. Higher concentrations at 100 μg/l and above, other organs such as olfactory pits and skin (Fig. 3.28A) were also observed to express GFP but with weak intensity and no apparent dosage dependence.

Preliminary 72 exposure hours of sodium arsenate exposure to 1-3 dph wild type fry had no mortality observed at concentrations of 10 μg/l to 50 μg/l, but at 100 μg/l and above, high mortality rate of at least 62.5% were observed. We treated the Tg(*hsp70:gfp*) fry (1-3 dph) with sodium arsenate from 12.5 μg/l up to 200 μg/l for 24 hours to determine GFP expression. The lowest effective concentration of sodium arsenate for observation of visible GFP induction in this transgenic line (Fig. 3.29A) was between 12.5 and 25.0 μg/l because 26% of the fry in 25.0 μg/l showed GFP induction in the liver but no expression was observed at 12.5 μg/l (Fig. 3.29B). In concentrations from 50 to 200 μg/l, all fry were observed to express GFP in the liver (Fig. 3.29A). Besides that, there was an increasing numbers of fry expressing GFP in other organs such as gut, muscle and skin when dosage increased. Generally, the GFP expression in most organs was also intensified in most fry when exposed to higher concentration of sodium arsenate.

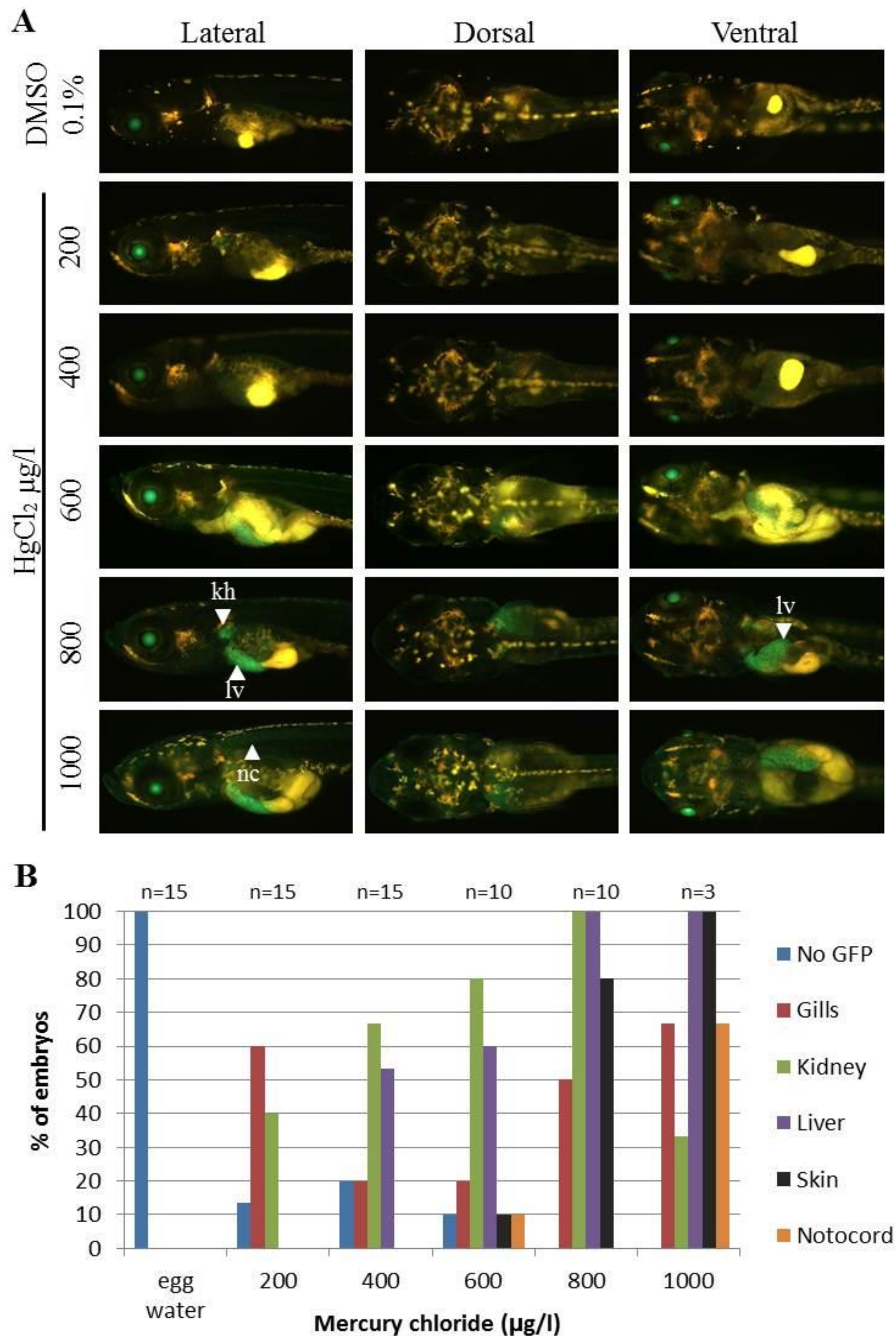
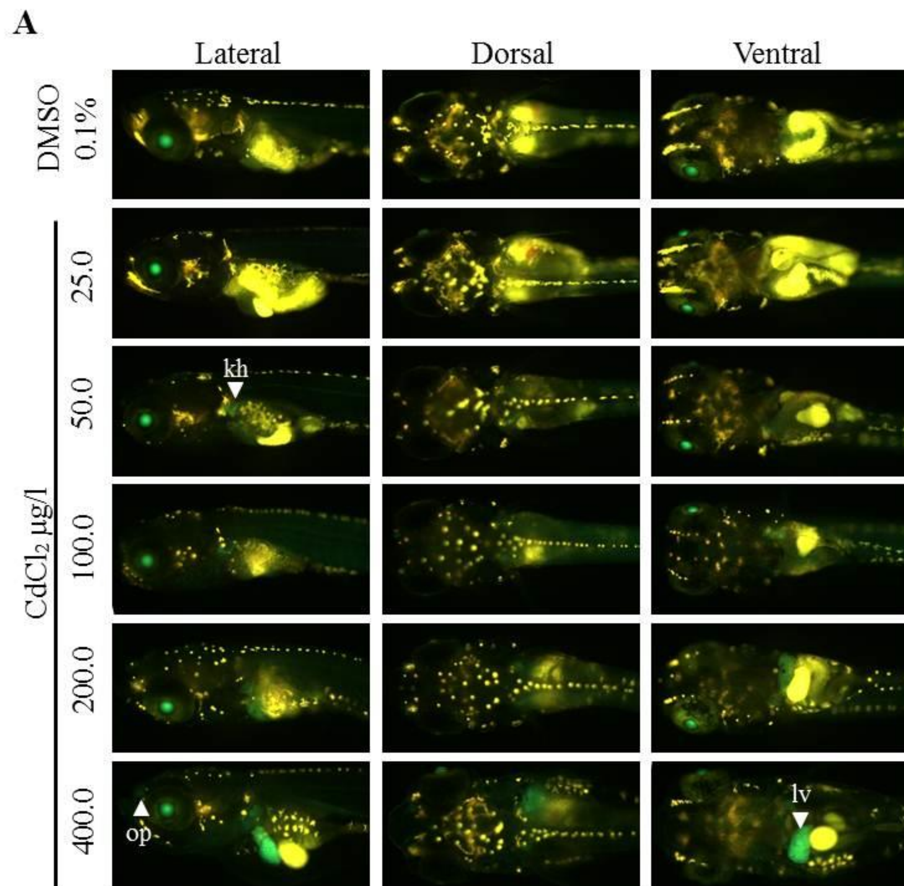


Figure 3.27 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of mercury chloride. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. White arrow heads demonstrate the position of various organs. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in different organs after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: kh, kidney head; lv, liver; nc, notochord.



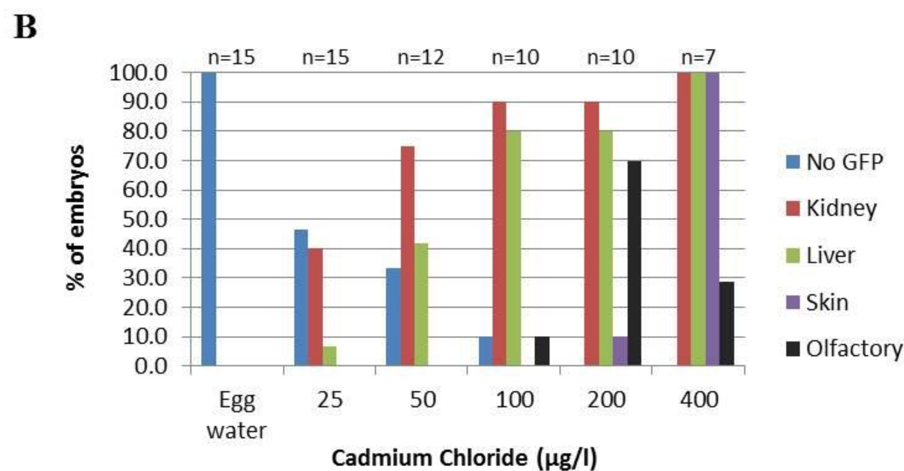
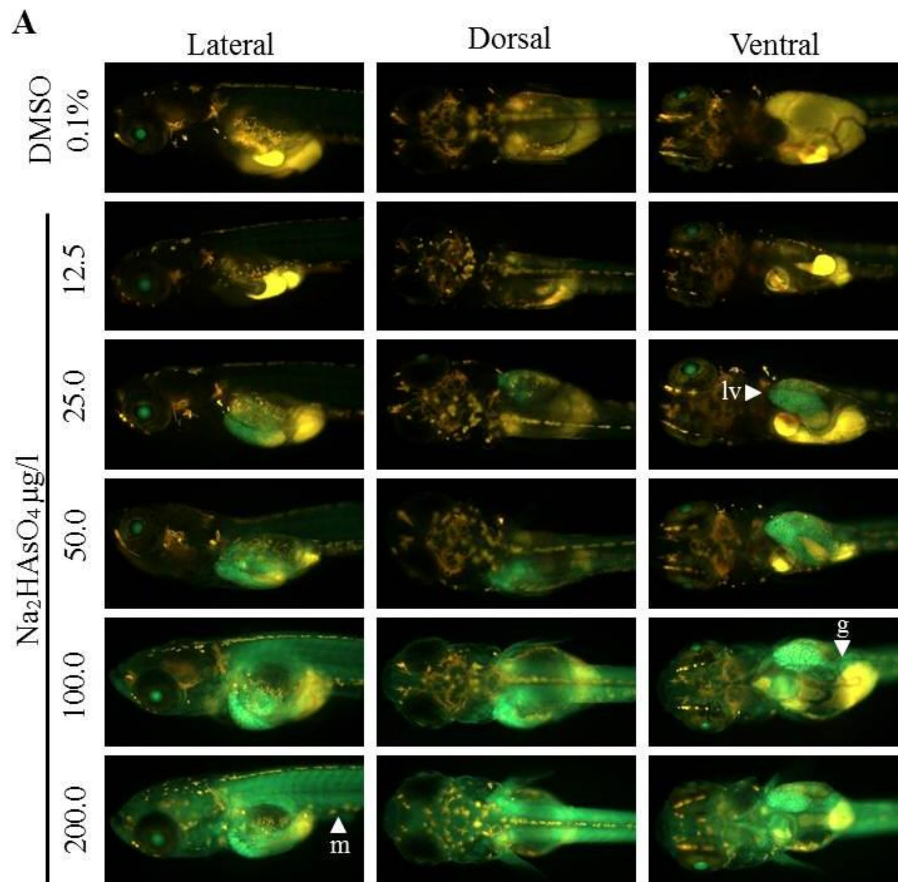


Figure 3.28 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of cadmium chloride. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. White arrow heads demonstrate the position of various organs. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in different organs after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: kh, kidney head; lv, liver; op, olfactory pits.

A



B

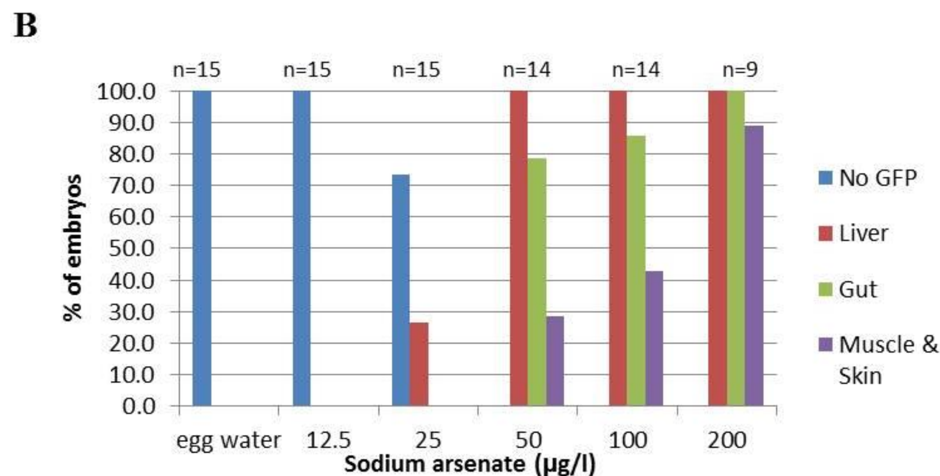


Figure 3.29 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of sodium arsenate. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. White arrow heads demonstrate the position of various organs. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in different organs after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: g, gut; lv, liver; m, muscle.

3.4.2 Exposure of Tg(*hsp70:gfp*) fry to other pollutants

Tg(*hsp70:gfp*) fry were also treated with other categories of chemical listed in Fig 1.1 to examine their inducibility of GFP expression in various classes of possible pollutants. These chemicals included 4-nitrophenol, bisphenol A, TCDD and lindane. Prior to chemical treatment with transgenic lines, preliminary exposure of wild type 1-3 dph fry was performed for each chemical for 72 hours. These observations were summarized in Table 3.3 for various chemical exposures.

For 4-nitrophenol (Fig. 3.30), bisphenol A (Fig. 3.31) and TCDD (Fig. 3.32), no GFP expression was induced in the transgenic fry even after 48 hours of exposure. We noted that the highest concentrations, 12.5 mg/l and 10 mg/l, used for 4-nitrophenol and bisphenol A respectively were lethal and all the fry in these groups died within 48 hours and no GFP expression from these fry was observed at any time.

In lindane treatment, no obvious GFP expression was initially observed in any organ (Fig. 3.33A). However, after 48 hours exposure, some of the fry in 1-mg/l and 5-mg/l lindane-treated groups showed weak and discrete GFP expression in the body trunk (Fig. 3.33B). GFP expression appears to be in the individual muscle fibers of the body trunk. Such observation accounts for 17.6% and 50% of the fry exposed in 1 mg/l and 5 mg/l concentration group, respectively (Fig. 3.33C).

Table 3.3 Observations of preliminary 72 hrs exposure of chemicals from various classes of pollutants using 1-3 dph wild type fry.

Chemical	Dosage used	Observations
4-nitrophenol	2.5 to 15.0 mg/l (17.97 μM to 107.83 μM)	High mortality rate, above 75%, was observed in embryos treated with 12.5 mg/l or higher of 4- nitrophenol. No mortality rate was observed for concentrations below that of 12.5 mg/l 4-nitrophenol.
Bisphenol A	0.1 to 10.0 mg/l (0.44 μM to 43.80 μM)	Mortality above 75% was observed in 10 mg/l of bisphenol A-treated embryos but no mortality was observed at lower concentrations.
TCDD	0.03 to 1.61 μg/l (0.1 to 5.0 nM)	No death was observed in any concentrations of TCDD, even at the highest concentration, 1.61 μg/l of TCDD. However, bended tail morphology was observed in some fry in TCDD from 0.03 μg/l to 1.61 μg/l and the number of fry with such morphology increased with dosage.
Lindane	0.68 to 12.5 mg/l (2.32 μM to 42.98 μM)	High mortality rate (>75%) was observed in concentrations at ≥ 5 mg/l of lindane treated embryos while low mortality rate (< 20%) was observed in 2.5 mg/l of lindane treated embryos and the concentrations below 2.5 mg/l had no recorded death. However, deformities such as crooked and shrunken body trunk and swimming difficulties were observed in some fry in lindane concentrations from 0.675 mg/l up to 10 mg/l. As the dosage increased, the number of fry with such deformities increased and the extent of deformities became more pronounced.
Mercury chloride	100 to 1000 μg/l (0.37 μM to 3.68 μM)	High mortality rate of 87.5% was observed in 1000 μg/l mercury chloride-treated group while lower mortality rates, 12.5% to 37.5%, were observed in concentrations at 400 μg/l to 800 μg/l of mercury chloride. No death were recorded for 200 μg/l and below of mercury chloride treatment.

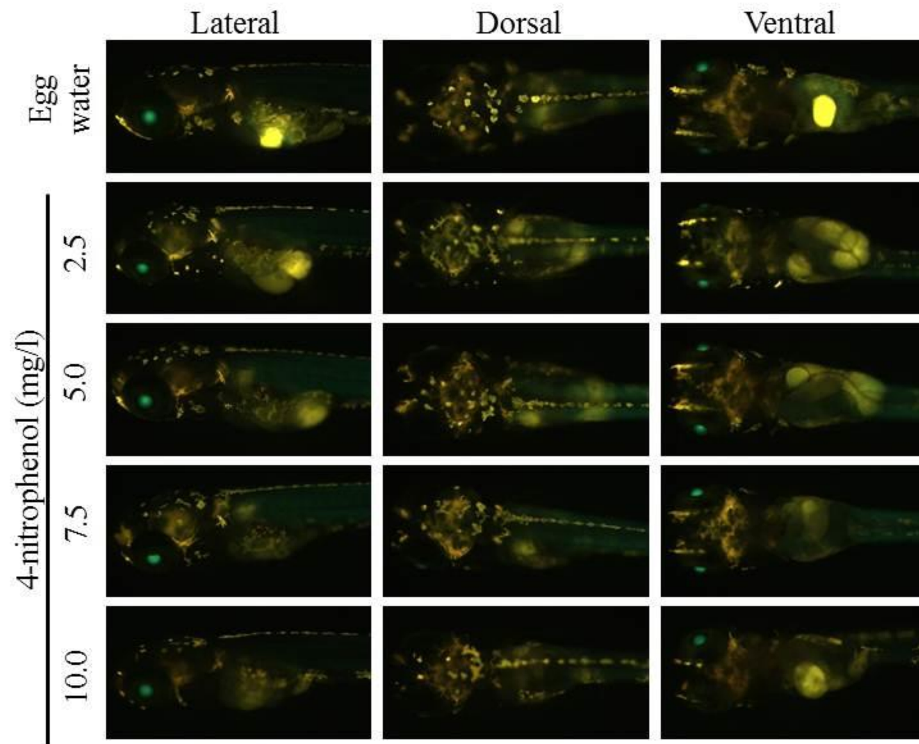


Figure 3.30 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentration of 4-nitrophenol. Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder.

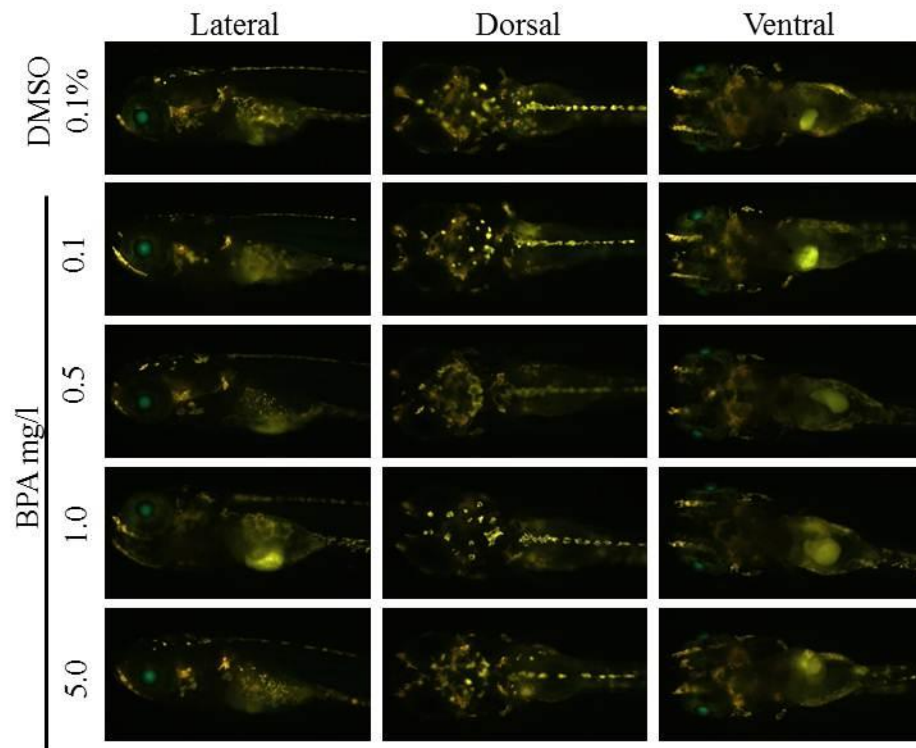


Figure 3.31 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentration of BPA. Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder.

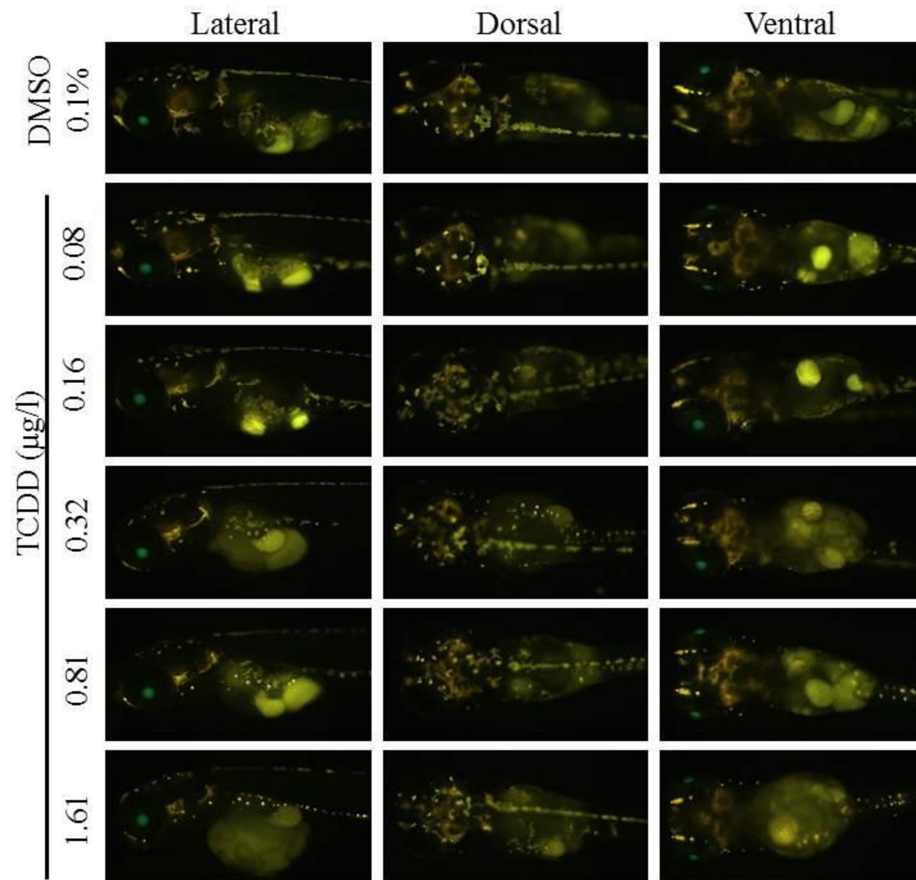
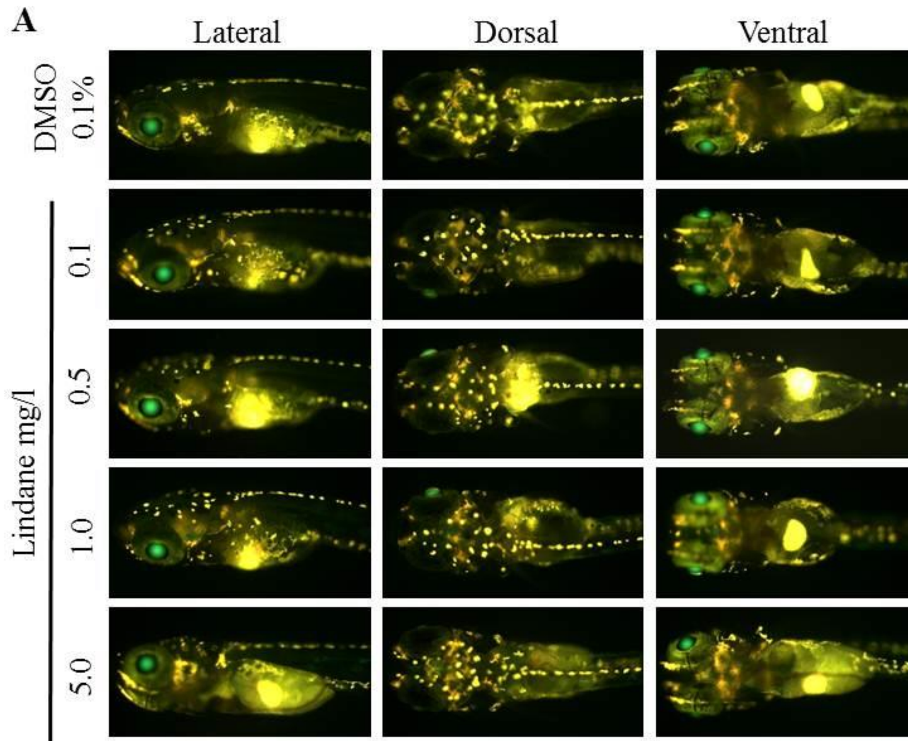
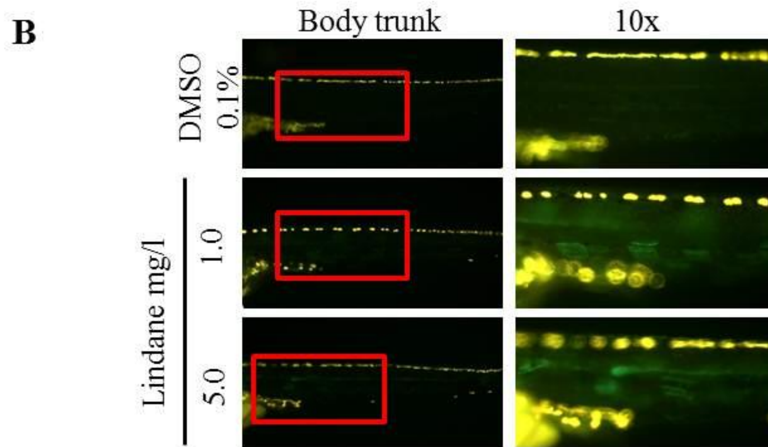


Figure 3.32 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentration of TCDD. Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder.


A
Lateral
Dorsal
Ventral
DMSO 0.1%
Lindane mg/l
0.1
0.5
1.0
5.0


B
Body trunk
10x
DMSO 0.1%
Lindane mg/l
1.0
5.0

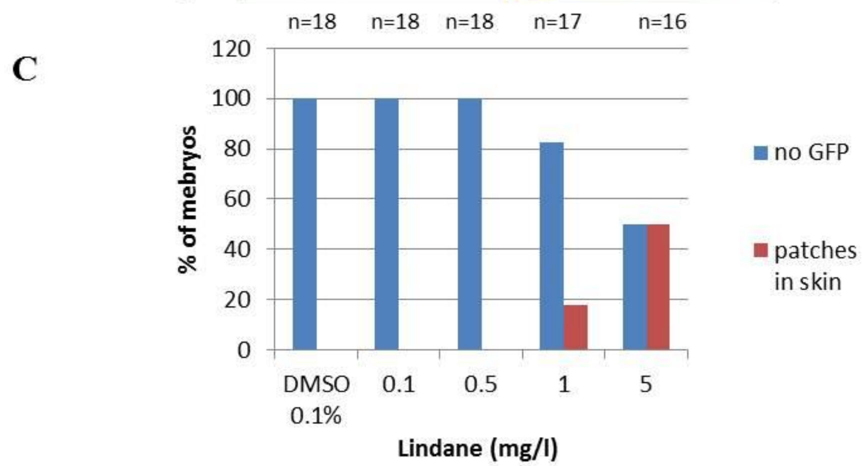

C
n=18
n=18
n=18
n=17
n=16
% of mebryos
120
100
80
60
40
20
0
DMSO 0.1%
0.1
0.5
1
5
Lindane (mg/l)
no GFP
patches in skin

Figure 3.33 Exposure of Tg(*hsp70:gfp*) 1.1 fry to various concentrations of lindane. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder. (B) Higher magnification of GFP induction observed in the body trunk of transgenic fry after 24 hours of treatment in 1 mg/l and 5 mg/l. Red boxes in the left column outline the area enclosed with 10x magnification view in the right column. (C) Histogram summary of the percentage of fry that showed patchy GFP induction in skin after treatment. The total numbers of fry used are indicated above each concentration group.

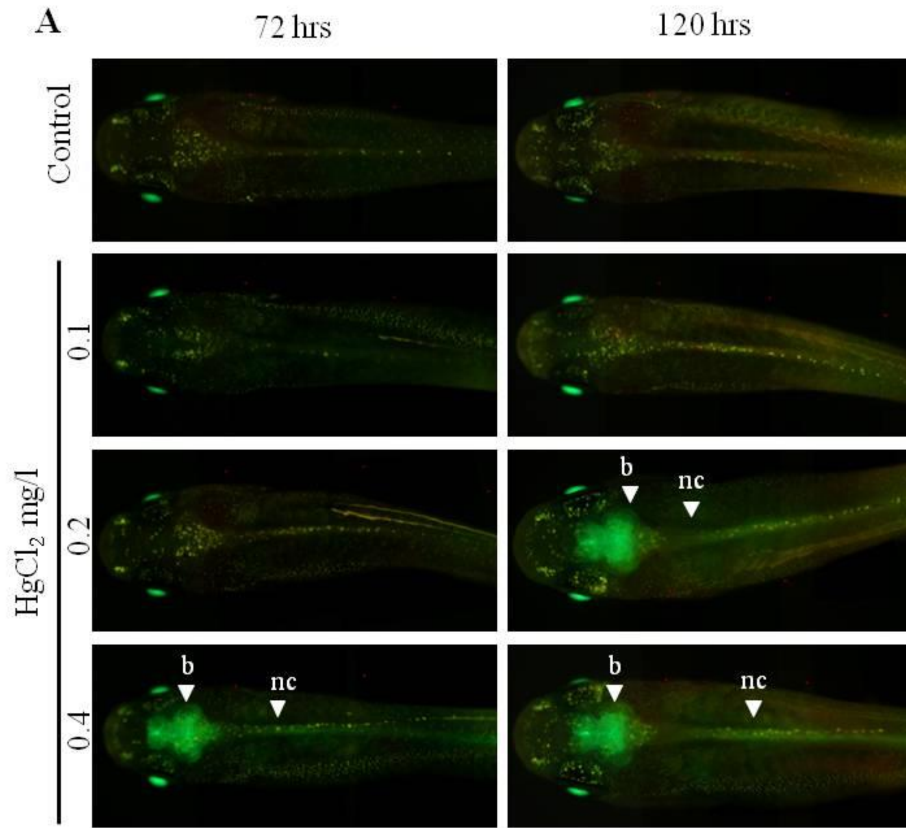
3.4.3 Mercury exposure of adult Tg(*hsp70:gfp*) fish

It would be more practical if adult fish could be used as on-site surveillance tool. However, adult fish body wall would not be as translucent as those in the larvae stage, hence posing difficulties in detecting GFP signal especially from internal organs. Nevertheless, we would like to test the adult fish for visual detection of GFP externally. Tanks with various concentrations of mercury chloride (0.1 mg/l, 0.2 mg/l and 0.4 mg/l and a water control) were set up. Each tank contained five male and five female Tg(*hsp70:gfp*) 6 months-old fish in static exposure conditions for five days. Two male and two female fish were randomly selected from each tank on time point at 1 day, 3 days and 5 days of exposure, for viewing of GFP expression under a fluorescent stereomicroscope before returning them back to their tanks.

GFP expression was not observed externally after 24 hours of mercury chloride exposure. After 72 hours of exposure, GFP expression was observed in the brain region of the four randomly selected fish at the concentration of 0.4 mg/l mercury chloride (Fig. 3.34A). After 120 hours of exposure, seven out of ten fish died at 0.4 mg/l mercury chloride while all ten fish survived for control, 0.1 mg/l and 0.2 mg/l mercury chloride. GFP expression was observed in the brain region of 5 out of 10 fishes in 0.2 mg/l (Fig. 3.34B). All three surviving fish in 0.4 mg/l mercury chloride had the similar GFP expression in the brain region but fish in control and 0.1 mg/l mercury chloride groups showed no GFP expressions. Dissections of the surviving fish were performed to view the GFP induction in the internal organs (Fig. 3.34B). As mentioned in Section 3.3.4.1, female fish had constitutive GFP expression in ovary as shown in water control. GFP expression was also induced weakly in the kidney and discretely in the liver but moderately in the gut of the three surviving fish in 0.4 mg/l mercury chloride. In the 0.2 mg/l mercury chloride treatment group, six

out of ten fish were observed to express GFP weakly in the kidney and moderately in the gut. GFP expression was also observed weakly in the kidney in four out of ten fish treated with 0.1 mg/l mercury chloride.

The observations obtained from the adult fish exposure suggested that GFP was prominent in the brain region externally upon induction and were visible by the third day of exposure. Kidneys appeared to be more sensitive to low concentration of mercury chloride at 0.1 mg/l, however were not visible externally unless dissection was performed.


A
72 hrs
120 hrs
Control
HgCl2 mg/l
0.1
0.2
0.4
b
nc
b
nc
b
nc

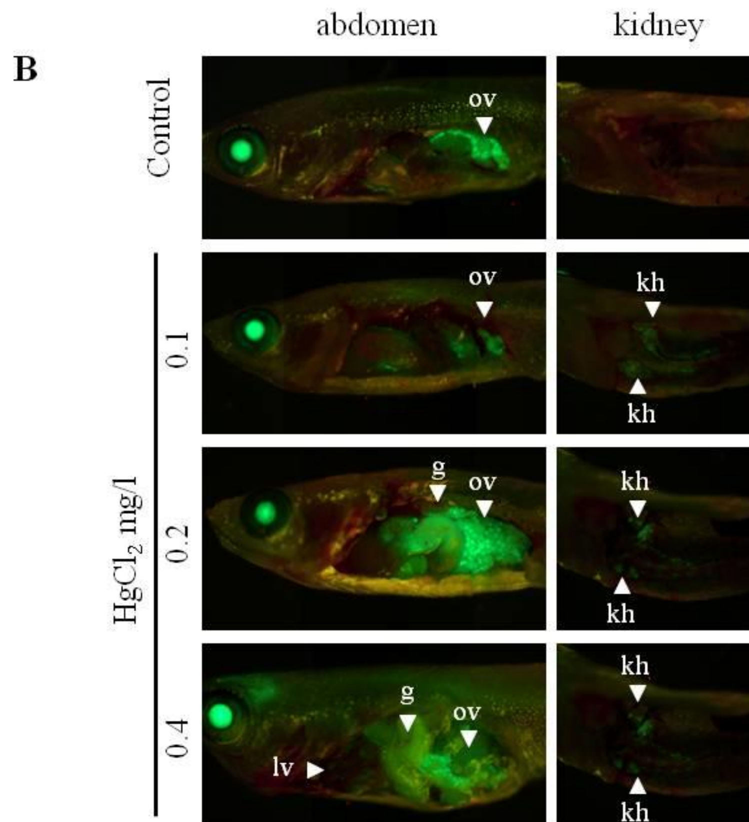

B
abdomen
kidney
Control
HgCl2 mg/l
0.1
0.2
0.4
ov
ov
kh
kh
g
ov
kh
kh
g
ov
lv
kh
kh

Figure 3.34 Mercury exposure of adult Tg(*hsp70:gfp*) fish. (A) Lateral views of GFP induction of a representative female fish from each concentration group at 72 hrs (left) and 120 hrs (right) after mercury exposure. (B) GFP expression in internal organs of the fish dissected after 5 days of exposure. Abbreviations: b, brain; g, gut; kh, kidney head; lv, liver; nc, notochord; ov, ovary.

3.4.4 Conclusion of Tg(*hsp70:gfp*) as a biomonitoring fish

Table 3.4 Summary of GFP induction of Tg(*hsp70:gfp*) fry by various chemical exposure

Chemicals	GFP expression	Lowest effective concentration	EPA MCL* (µg/L)
Mercury chloride	liver, kidney, notochord, gills and skin	≤200 µg/l (736.6 nM)	2.0
Cadmium chloride	kidney, liver, olfactory pits and skin	≤25 µg/l (136.4 nM)	5.0
Sodium arsenate	liver, gut, muscle and skin	12.5 - 25 µg/l (40.1 -80.1 nM)	10.0
4-nitrophenol	No GFP induction	Nil	nil
Bisphenol A	No GFP induction	Nil	nil
TCDD	No GFP induction	Nil	0.00003
Lindane	Weak and discrete GFP in muscle fibers	0.5 - 1 mg/l (1.7 -3.4 µM)	0.2

*: EPA MCL-Maximum Contaminant Level (MCL) from EPA. (US Environmental Protection Agency, 2009)

Overall, Tg(*hsp70:gfp*) 1.1 was responsive towards various heavy metal salt such as mercury chloride, cadmium chloride and sodium arsenate, as summarized in Table 3.4. In these heavy metal treatments, generally, the liver was the common organ to induce GFP. However, there were some organs that induced GFP only in certain metal exposure with high concentrations, suggesting differential toxicity and/or accumulation difference of the chemicals. GFP in the notochord was observed only in the fry treated with mercury chloride while GFP could be seen in the olfactory pits after exposure to cadmium chloride. Strong GFP was observed in the body trunk of the sodium arsenate treated fry. Using 6 months-old adult fish for mercury chloride exposure, GFP expression was also observed externally by third day although the expression pattern of the organs deviated from that in the larvae stage treatment.

No GFP was induced in the transgenic fry by several other chemicals such as TCDD, 4-nitrophenol and BPA, although lethal concentrations and longer exposure period were used. This indicated that the Tg(*hsp70:gfp*) had rather specific response mainly to the metal group of chemicals with the exception of lindane, which only at high concentrations caused weak GFP expressions in few discrete muscle fibers of Tg(*hsp70:gfp*) after 48 hours of exposure. Thus, this Tg(*hsp70:gfp*) could be useful generally for detection of heavy metal pollutants.

3.5 Characterization of Tg(*cyp1a:gfp*) for biomonitoring application

3.5.1 PAH exposure of Tg(*cyp1a:gfp*) fry

Polycyclic aromatic hydrocarbons were known to up-regulate *cyp1a* gene via aryl hydrocarbon receptor (AhR) pathway. Therefore, Tg(*cyp1a:gfp*) fry were exposed to three chemicals, 3-methylcholanthrene (3-MC), benzo[a]pyrene (BAP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which are known to increase *cyp1a* transcription upon exposure, for 24 hours. Prior to chemical exposure to Tg(*cyp1a:gfp*) 4.2 fry, preliminary tests with these PAHs were performed with wild type 1-3 dph fry for 72 hours. No mortality was observed even with considerably high concentration of BAP (1000 µg/l), 3-MC (1000 µg/l) and TCDD (1.61 µg/l or 5 nM). Perhaps, their toxicity was chronic instead of acute as their major known effect is carcinogenic. However, bended tail morphology was observed in some fry in TCDD from 0.03 µg/l (0.1 nM) to 1.61 µg/l (5 nM) and the number of fry with such morphology increased with dosage.

As shown in Figure 3.35A, GFP expression was observed intensely in the liver in all 3-MC treated groups of Tg(*cyp1a:gfp*) fry. GFP expression in the kidney and gut was weak at the low concentration of 6.25 µg/l but increased its green

fluorescence intensity as the dosage increased. At 100 µg/l 3-MC treated group, some of the fry were also observed to have weak GFP expression in the tail fin. As summarized in Fig. 3.35B, all fry that had GFP expression, had GFP induction in the liver after 3-MC exposure. A high percentage of those transgenic fry that had GFP induction would also induced GFP induction in the kidney since the percentage of GFP observed in the kidney was close to those observed in liver, with the exception of 6.25 µg/l group. However, for GFP expression in the gut, this observation only accounted for 30% of the total fry in each of the various concentrations of 3-MC treated groups regardless of the percentage of total fry that had GFP induction.

In all the BAP treated group, GFP expression was observed strongly in the liver, kidney and weakly in the gut (Fig. 3.36A), though the intensity of GFP expression was not dosage dependent. At 1000 µg/l, GFP was observed very weakly around the growing section of the tail fin for most of the fry. From Fig. 3.36B, all fry that expressed GFP would express in the liver. Furthermore, most of the fry that had induced GFP expression would most probably expressed GFP in the kidney as well as in the gut since the percentage of GFP expression in both organs were close to those percentages of GFP expression in the liver for all BAP concentrations (Fig. 3.36B).

In Figure 3.37A, liver and kidney seemed to be the most sensitive organs towards TCDD as at low concentration of 3.22 ng/l (0.01 nM), only these two organs expressed weak GFP in about 50% of the treated fry (Fig. 3.37B). At the concentrations of 16.1 ng/l (0.05 nM) and higher, 100% of the fry had GFP expression in the liver and kidney. As the dosage increased, GFP expression in other organs such as gut, mast cells, gills, tail fins, olfactory pits and sometimes blood vessels were also observed (Fig. 3.25F, I, L and Fig. 3.37A). The intensity of expression in all organs appears to be dosage dependent, with the maximum intensity

observed at 161 ng/l (0.5 nM) and 322 ng/l (1 nM). At 322 ng/l (1 nM), apparently the expression was saturated with all fry expressing strong GFP signal in all organs.

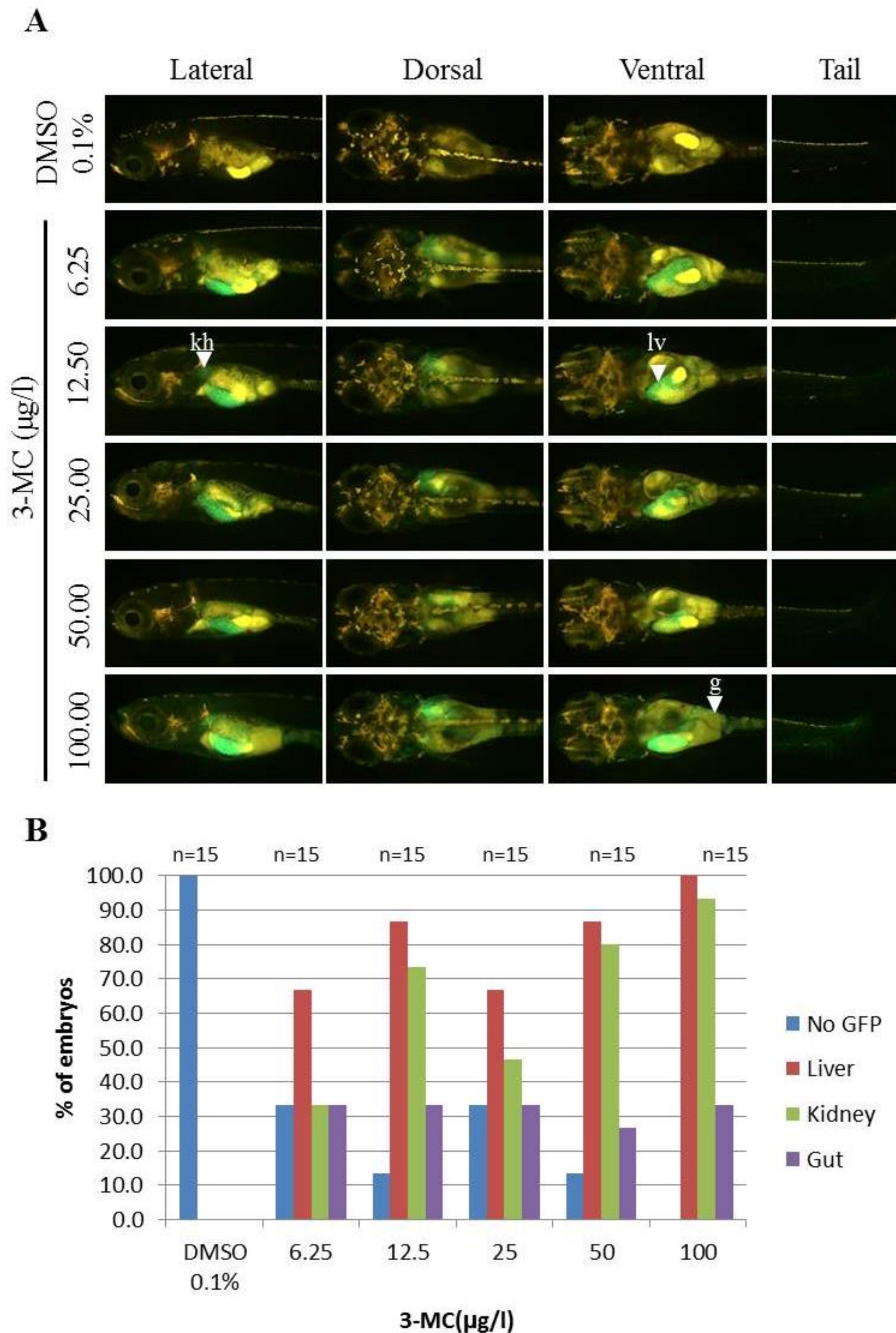
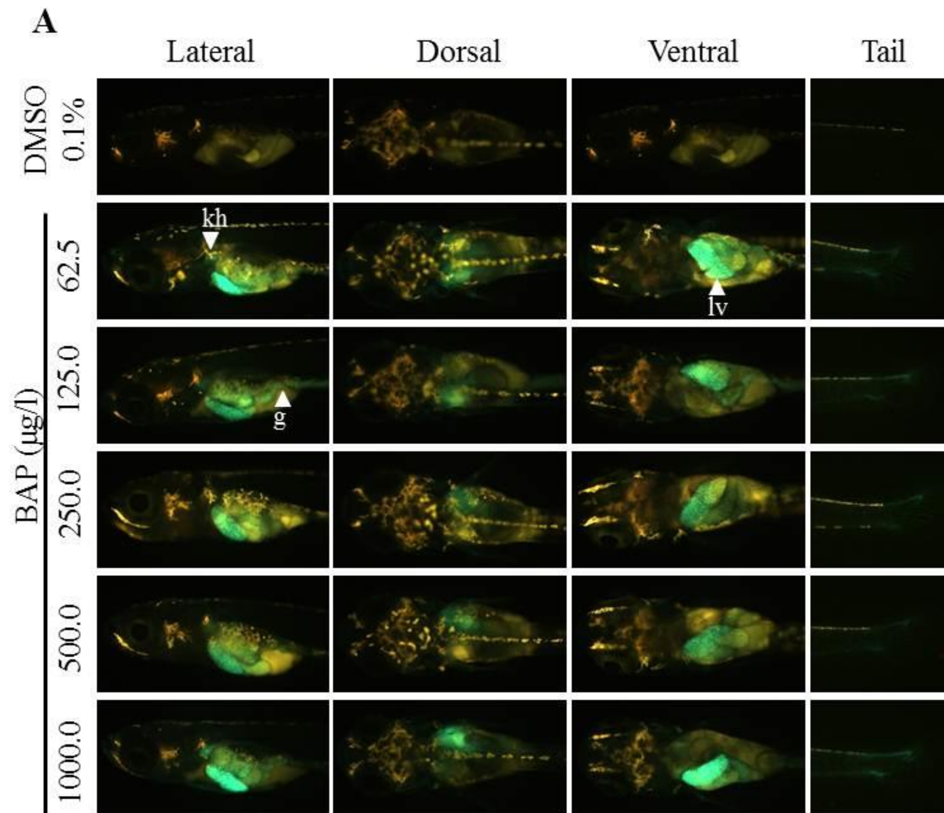


Figure 3.35 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of 3-MC. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. White arrow heads demonstrate the position of various organs. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in different organs after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: kh, kidney head; lv, liver; g, gut.



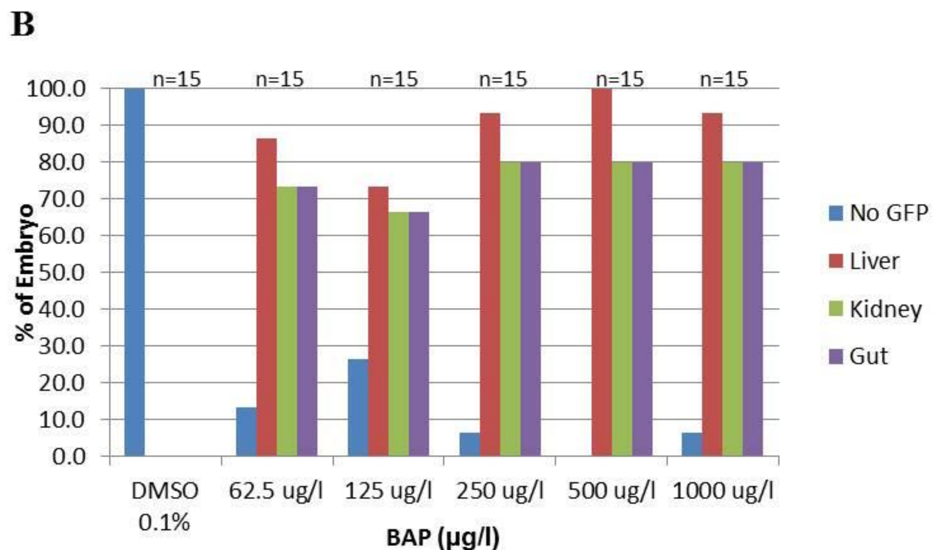


Figure 3.36 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of BAP. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. White arrow heads demonstrate the position of various organs.Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in different organs after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: kh, kidney head; lv, liver; g, gut.

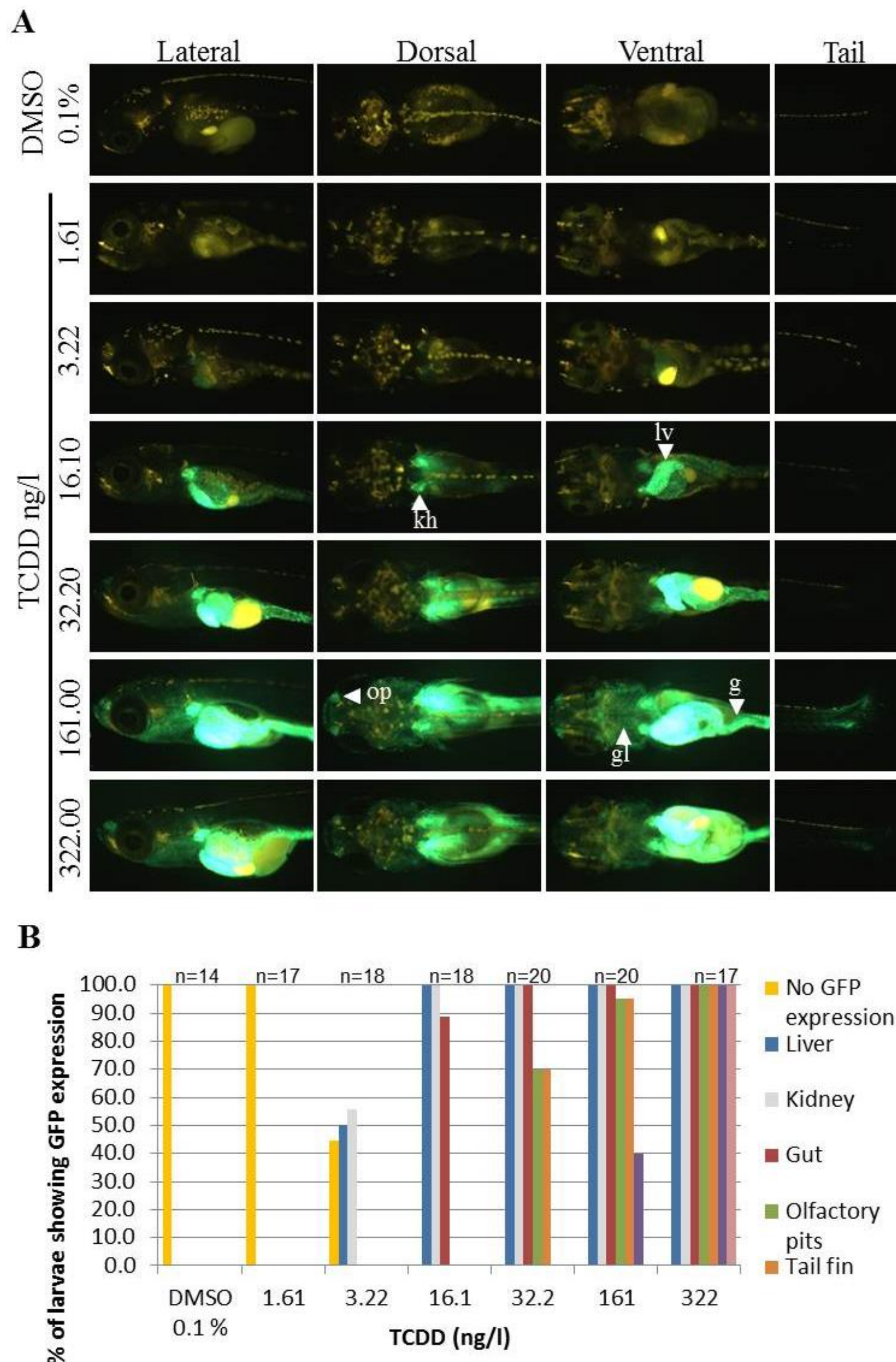


Figure 3.37 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of TCDD. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. White arrow heads demonstrate the position of various organs. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in different organs after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: kh, kidney head; lv, liver; g, gut; gl, gills; op, olfactory pits.

3.5.2 Exposure of Tg(*cyp1a:gfp*) fry to other pollutants

Tg(*cyp1a:gfp*) fry were also treated with other categories of chemical listed in Fig 1.1 to examine their inducibility of GFP expression in various classes of possible pollutants. These chemicals include 4-nitrophenol, bisphenol A, mercury chloride and lindane. Prior to chemical treatment with transgenic lines, preliminary exposure of wild type 1-3 dph fry was performed for each chemical for 72 hours. The observations of these chemical exposures were summarized in Table 3.3.

No GFP expression was observed in Tg(*cyp1a:gfp*) fry (1-3 dph) treated with bisphenol A (Fig. 3.38), mercury chloride (Fig. 3.39) and lindane (Fig. 3.40) even after 48 hours of exposure. We noted that the highest concentrations used for 4-nitrophenol, bisphenol A and lindane were lethal and all the fry in these groups died within 48 hours yet no GFP expression of these fry was observed at any time.

Spotty GFP expression (Fig 3.41A) was observed in the livers of a few fry treated with two high concentrations of 4-nitrophenol used: 5 out of 29 fries (17.2%) at 7.5 mg/l and 6 out of 29 fries (20.7%) at 10 mg/l (Fig. 3.41B). No GFP expression was observed in fry at lower concentrations of 4-nitrophenol.

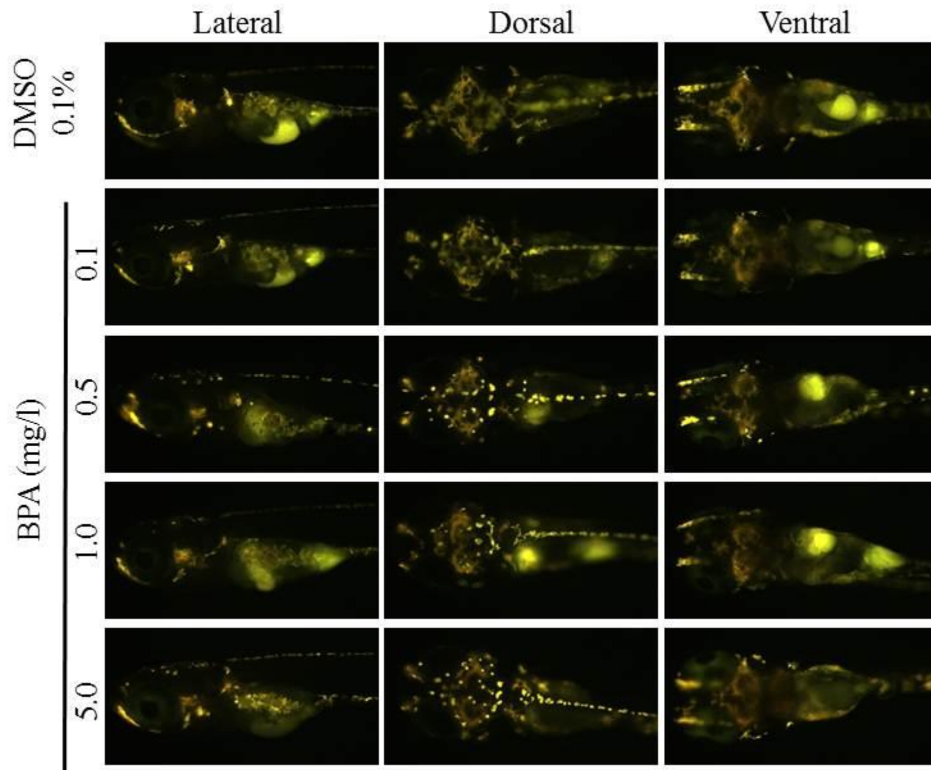


Figure 3.38 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of BPA. Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder.

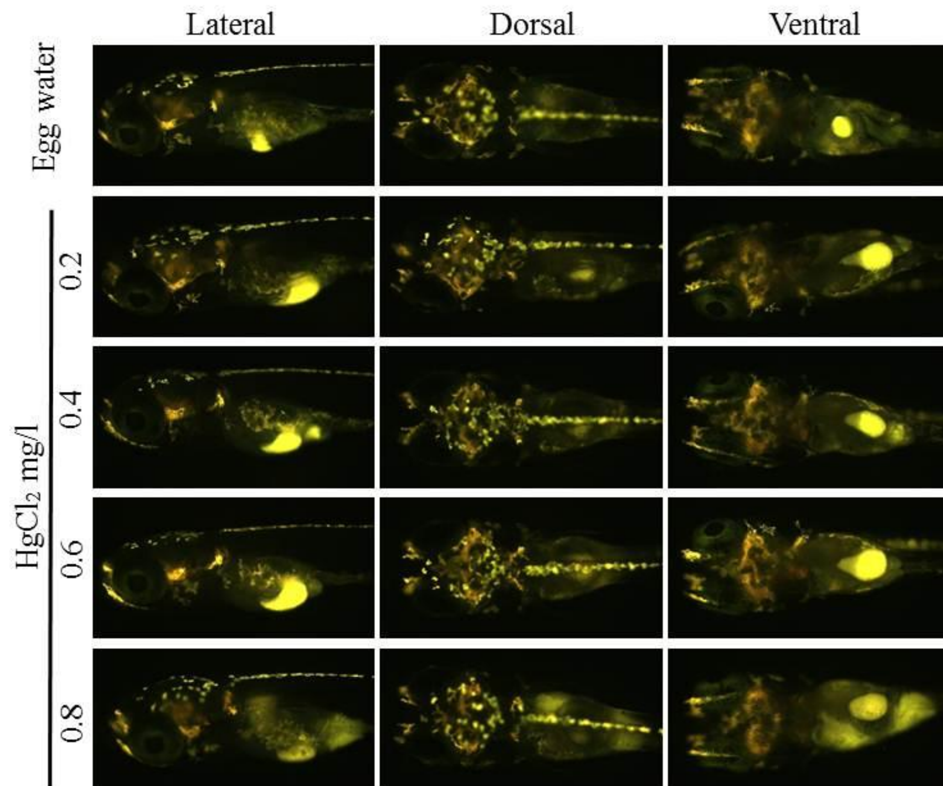


Figure 3.39 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of mercury chloride. Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder.

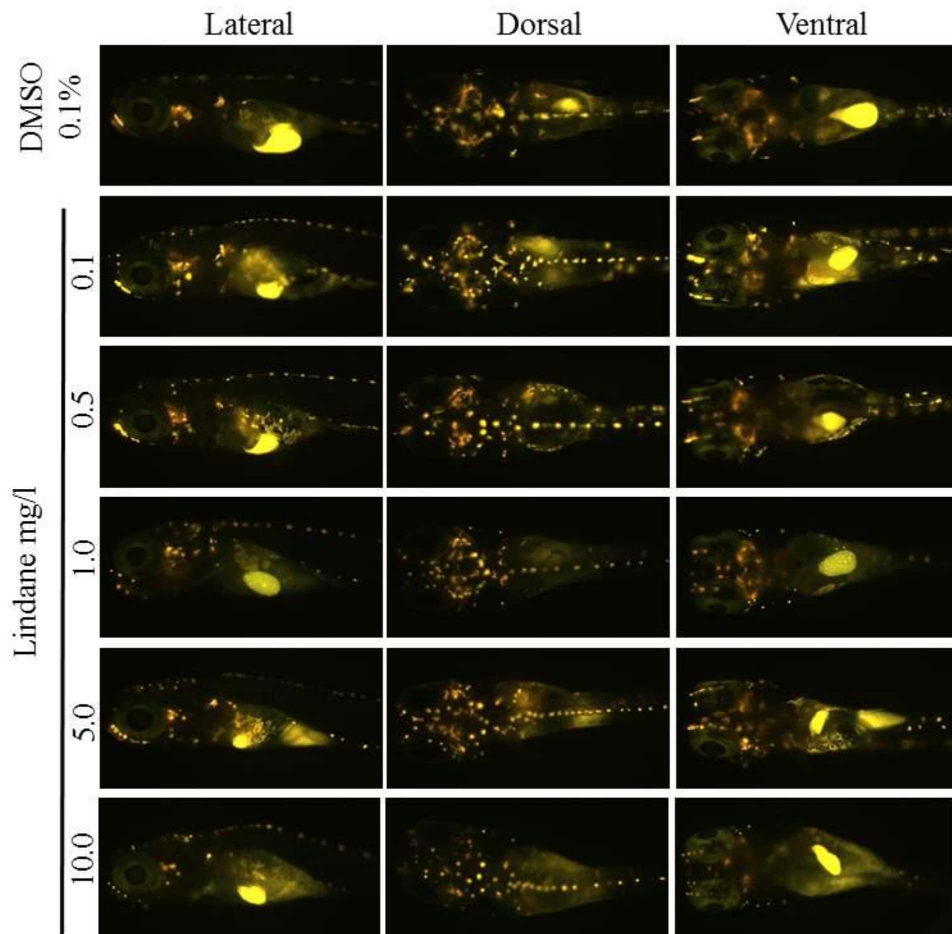


Figure 3.40 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of lindane. Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto-fluorescence of pigment cells, yolk and gall bladder.

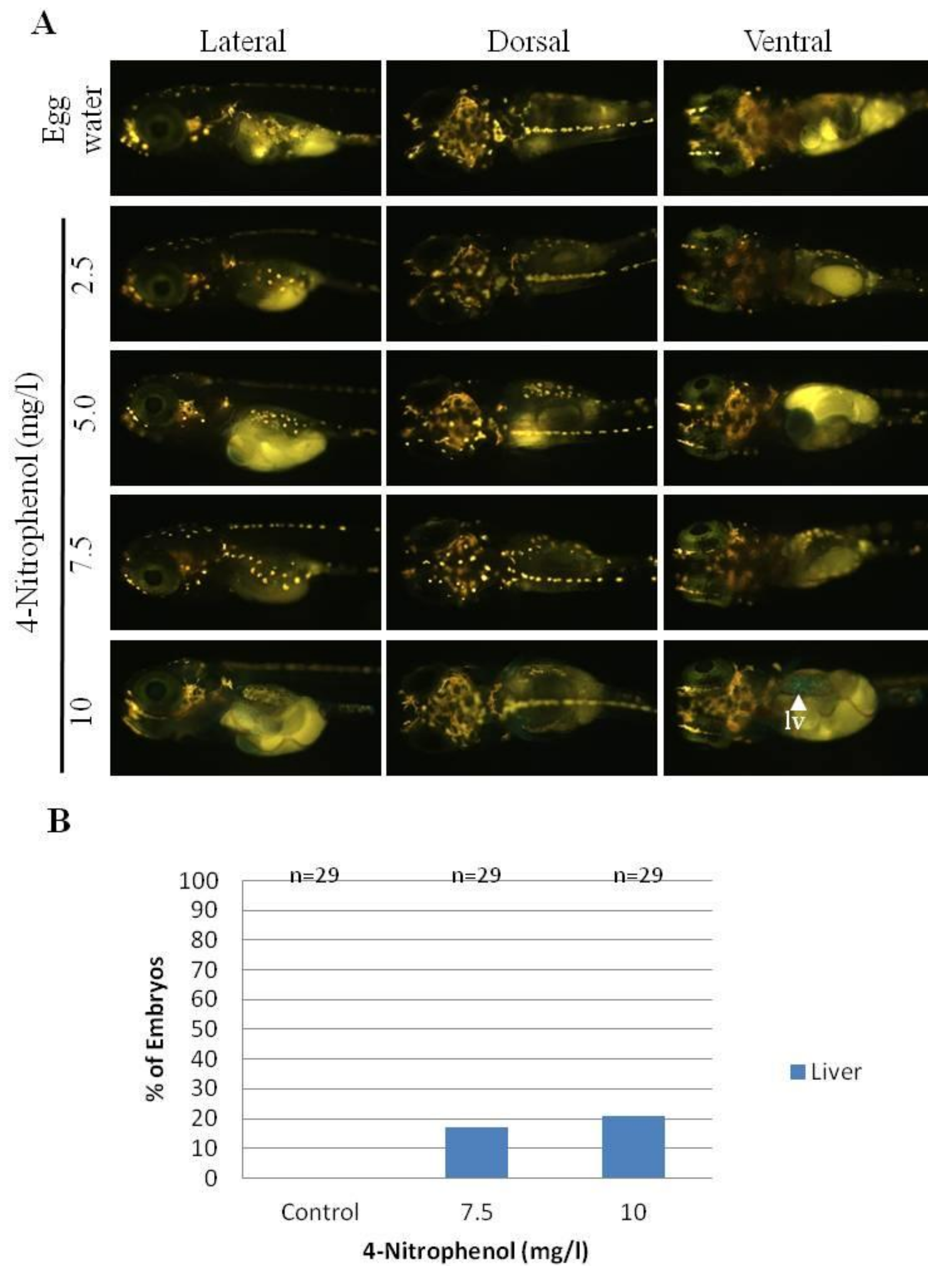


Figure 3.41 Exposure of Tg(*cyp1a:gfp*) 4.2 fry to various concentrations of 4-nitrophenol. (A) Lateral, dorsal and ventral views of representative transgenic fry after 24 hours of treatment in different concentration groups in the same set of experiment. Yellow regions observed were auto fluorescence of pigment cells, yolk and gall bladder. (B) Histogram summary of the percentage of fry that showed GFP induction in liver after treatment. The total numbers of fry used are indicated above each concentration group. Abbreviations: lv, liver.

3.5.3 TCDD exposure of adult Tg(*cyp1a:gfp*) fish

Similar to Tg(*hsp70:gfp*), 4-month-old adult fish of Tg(*cyp1a:gfp*) were used for assessment of visual detection of GFP induction. This was conducted by treating adult Tg(*cyp1a:gfp*) fish with various concentrations of TCDD: 32.2 ng/l (0.1nM), 161.0 ng/l (0.5nM) and 805 ng/l (2.5nM) as well as vehicle solvent control (0.1% DMSO) in static exposure conditions for three days. Each concentration group contained five male and five female Tg(*cyp1a:gfp*) fish. Two male and two female fish were randomly selected from each tank after 1 and 3 days of exposure to view GFP expression under a fluorescent stereomicroscope before returning them back to their tanks.

After 1 day of treatment, the four randomly selected fish were observed to express GFP in the kidney region, urinary pore and olfactory pits in all TCDD treated fish but not in the DMSO vehicle control group (Fig. 3.42A). After 3 days of exposure, all fish from TCDD-treated groups in all three concentrations were observed to express GFP in head, trunk, skin and brightly in head kidney, urinary pores, gills region, mast cells around the eyes, lips and olfactory pit as well as rib cage (Fig. 3.42A). No mortality was observed in this experiment. We dissected all the fish to view its internal organs (Fig. 3.42B). In all TCDD treatment groups, the liver was found to have the most intense GFP signal followed by the gut. The GFP signal was also strong in the head kidney and kidney tubules for all fishes in all TCDD treatment groups. Interestingly, the abdomen wall that was dissected out did not fluorescence after dissection. This indicated that the earlier GFP signal seen in the rib cage was probably due to the strong signal from the internal organs which illuminated the rib cage.

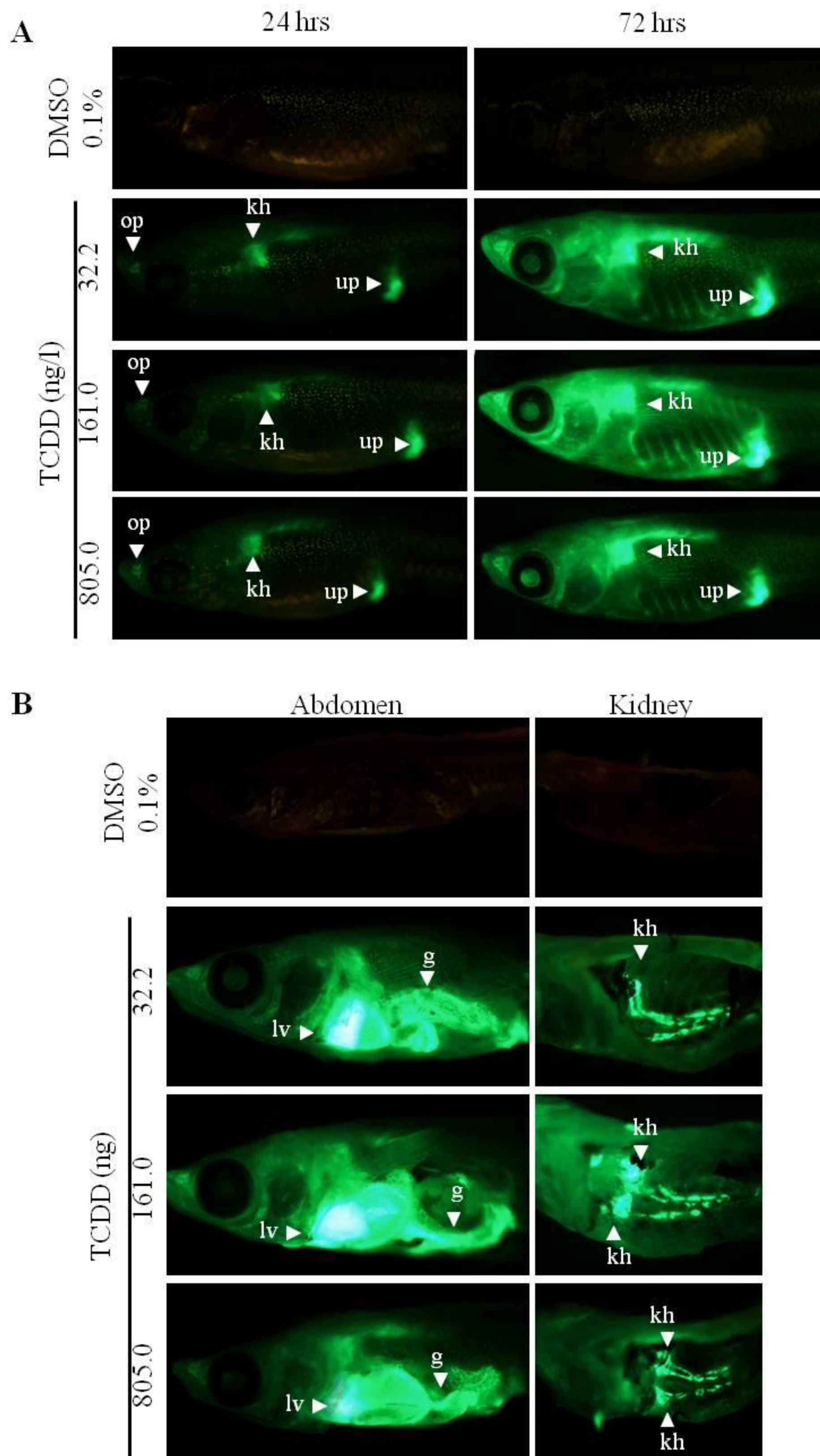


Figure 3.42 TCDD treatment of adult Tg(*cyp1a:gfp*) 4.2 fish. (A) Lateral views of GFP induction of a representative male fish from each concentration group at 24 hrs (left) and at 72 hrs (right) after TCDD exposure. (B) GFP expression in internal organs of the fish dissected after 72 hrs of exposure. g, gut; kh, kidney head; lv, liver; op, olfactory pits; up, urinary pore.

3.5.4 Conclusion of Tg(*cyp1a:gfp*) as biomonitoring fish

Table 3.5 Summary of GFP induction in Tg(*cyp1a:gfp*) fry by various chemical exposures.

Chemicals	GFP expression	lowest effective concentration	EPA MCL µg/l
3-MC	liver, kidney and gut	≤ 6.25 µg/l (23.3 nM)	Nil
BAP	liver, kidney and gut	≤ 62.5 µg/l (247.7 nM)	0.2
TCDD	liver, kidney, gut, olfactory pits, gills, blood vessels, mast cells	1.61 - 3.22 ng/l (0.005 -0.01 nM)	0.00003
4-nitrophenol	Spotty GFP expression in liver	5.0 - 7.5 mg/l (35.9 -53.9 µM)	Nil
Bisphenol A	No GFP induction	Nil	Nil
Mercury chloride	No GFP induction	Nil	2.0
Lindane	No GFP induction	Nil	0.2

*: EPA MCL-Maximum Contaminant Level (MCL) from EPA. (US Environmental Protection Agency, 2009)

The responses of Tg(*cyp1a:gfp*) fry to all three PAHs, namely 3-MC, BAP and TCDD exposures were summarized in Table 3.5. Generally, GFP was commonly observed in the liver, kidney and gut for PAH exposure. However, in TCDD exposure, GFP was also induced in other organs including olfactory pits, gills, blood vessels and, at high concentration, mast cells. Similarly, TCDD treatment of adult transgenic fish also showed induction of intense GFP expressions, by 24 hours of exposure. No GFP expression was induced by other categories of chemicals such as bisphenol A, mercury and lindane, except for 4-nitrophenol where weak GFP expression was observed in approximately 20% of the fry at high concentration. Thus, this Tg(*cyp1a:gfp*) was quite specific to respond to PAH pollutants by GFP induction and had a potential to be developed as a valuable biomonitoring tool for PAHs.

DISCUSSION

4. Discussion

4.1 Selection of inducible promoters

It has been suggested to use transgenic fish for aquatic monitoring by using pollutant-inducible response elements in a reporter gene construct to generate transgenic fish (Carvan *et al.*, 2000). Examples of such response elements include those responsive to aromatic hydrocarbon, heavy metal, heat shock, oxidative stress etc. In the present study, we proposed to use two popular biomarker genes, *hsp70* and *cyp1a,* for our responsive promoters to generate environmental monitoring transgenic fish. We first analyzed the promoters for the presence of relevant response elements before using them to establish transgenic medaka lines.

4.1.1 *hsp70* promoter

The gene *hsp70* (Ensembl gene ID ENSORLG00000000233) was demonstrated to be up-regulated by both heat shock and mercury chloride treatment (Fig. 3.1) in medaka. These results are consistent with other studies on induction of *hsp70* by heat shock (Arai *et al.*, 1995) and by heavy metal exposure in aquatic organisms (Yoshimi *et al.*, 2009; Pinsino *et al.*, 2010). To construct hsp70-EGFP plasmid, we analyzed the promoter region of *hsp70* for relevant transcription regulatory elements to determine an appropriate region for cloning. The promoter sequence of endogenous medaka *hsp70* gene contains five putative HSEs, one MRE as well as one EpRE within 2 kb upstream of translation start codon of *hsp70* (Fig. 3.3). EpRE regulates the transcription via a primary transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), while HSE and MRE are the binding sites of heat shock factor (HSF) and MRE-binding transcription factor-1 (MTF-1) respectively. Oxidative stress caused by heavy metal exposure is thought to induce the transcription

of EpRE-regulated gene which has been demonstrated by EpRE-driven transgene expression in transgenic zebrafish after mercury exposure (Kusik *et al.*, 2008). There are reports of up-regulation of HSF and MTF-1 activated genes after heavy metals exposure in various organisms, indicating that HSEs and MREs are involved in response towards heavy metals insults (Huang *et al.*, 2007; Pinsino *et al.*, 2010; Liu *et al.*, 2001). The influence of overexpressed MTF-1 on heavy metal-induction of HSF1-dependent transcription in cell culture as well as supershift results of MTF-1 in HSF1/HSE complex implies that MTF and HSF regulatory pathway can crosstalk (Uenishi *et al.*, 2006). However, the exact signalling pathway involves in the up-regulation of *hsp70* by heavy metals is still vague. In addition, the putative TATA box is found to be far upstream (-623 bp) of medaka *hsp70* translation start codon. Since information regarding medaka *hsp70* transcription start site is not available, it is possible that transcription start site is near to putative TATA box since the TATA box is conventionally found within -30 to -20 bp of the transcription start site (Dikstein, 2011).

In the zebrafish *hsp70* promoter, there are six HSEs as well as a TATA box, CCAAT and GC elements located within the first 700 bp upstream of the ATG codon (Halloran *et al.*, 2000; Shoji & Sato-Maeda, 2008). Transgenic zebrafish with a 1.5-kb *hsp70* promoter has been shown to respond to heat shock as well as heavy metal stress (Halloran *et al.*, 2000; Blechinger *et al.*, 2002). Thus, 2 kb of medaka *hsp70* promoter is sufficient to drive the transcription under the stress condition including heat shock and metal exposure, as demonstrated in the present study.

4.1.2 *cyp1a* promoter

We have demonstrated that medaka *cyp1a* gene (ENSORLG00000014421) was up-regulated by TCDD and BAP, both of which are *cyp1a* inducers (Fig. 3.2). In our analysis of the medaka *cyp1a* promoter, we have searched for relevant important transcription regulatory or enhancer elements such as XREs, Sp1 binding site and TATA box. These elements have been frequently found in the promoters of xenobiotic inducible genes and are important for the transcription of *CYP1a* during induction. (Kawajiri & Fujii-Kuriyama, 2007; Fujii-Kuriyama & Mimura, 2005). The TATA and basic transcription element (BTE), in which Sp1 is the regulatory factor (Kobayashi *et al.*, 1996), is essential for promoter function. However, the functionality of *CYP1a* promoter is greatly influenced by the enhancer control consisting of cluster of XREs (Whitlock, 1999).

Previously, Kim *et al* (Kim *et al.*, 2004) has described promoter length of 2,263 bp of medaka *cyp1a* to contain six XREs, four MREs, one PRL motif, one Sp1 binding site and as well as the TATA box. One XRE is in distal region approximately 2000 bp downstream while the rest of XREs are in proximal region less than 700 bp downstream of transcription site. We used a more stringent consensus sequence, T/GNGCGTG, to search in both directions and five putative XREs are located instead (Fig. 3.4). The positions of the XREs in our analysis are not identical to Kim *et al.* 2004 but most of them are very similar in position. Similar to that of Kim *et al.* 2004, the position of TATA box is within expected region (about -25 to -35) (Dikstein, 2011). Perhaps due to different strains of medaka used, there is a slight difference in sequence identity (97.3%) between the two sequences. Kim *et al.* 2004 used HN1 strain but we used Hd-Rr strain for promoter cloning.

The analysis of zebrafish *cyp1a* promoter region (Zeruth & Pollenz, 2005; Zeruth & Pollenz, 2007) indicates eight putative XREs within 2.6 kb upstream of the transcription start site. The transcription of downstream gene is thought to be mediated by three out of these eight putative XREs since mutagenesis in these three XREs, located in distal region, greatly decreased the inducible response. European flounder *cyp1a* promoter region (1.3 kb) is analyzed to have eight potential XREs (Williams *et al.*, 2000). Similarly, through mutagenesis, three distal and one proximal XREs have been found to be essential for transcription efficiency during induction (Lewis *et al.*, 2004). Deletion of the distal region or mutation in the single proximal XRE of eel *cyp1a* promoter weakened the response significantly during induction (Ogino *et al.*, 1999). From these functional analyses of the *cyp1a* promoters, it is clear that not all XREs are functional and those that are functional are often located in the distal region. The spatial distance of the enhancer presumably helps to overcome the steric constraint so to mediate chromatin remodeling such that *cyp1a* promoter becomes more accessible to transcription factors (Whitlock, 1999). However, the replacement of the endogenous region between proximal cluster and distal cluster of XREs with another DNA sequence of similar length has shown reduced inducible response in the eel promoter functional analysis. This implies that the region between the distal cluster of XREs and proximal region of TATA, XREs may contain other elements to help stabilize the non-nucleosomal configuration during inducible condition (Ogino *et al.*, 1999). From our analysis of medaka *cyp1a* promoter, there is a distal cluster of two XREs and a proximal cluster of three XREs in addition to the TATA box and Sp1 binding site (Fig. 3.4). Thus the promoter length is sufficient to drive the inducible transcription as demonstrated in our transgenic analyses.

4.2 Use of maize Ac/Ds transposon system to generate transgenic medaka

Due to low germline transmission rate (<10%) achieved by conventional microinjection of plasmid into oocytes of the fish embryo (Zeng *et al.*, 2005; Miyamoto *et al.*, 2009; Zeng *et al.*, 2005; Kinoshita *et al.*, 2000), various technological tools such as meganuclease and transposon systems such as *Sleeping beauty* and *Frog prince* have been developed in recent years to improve transgenesis rate of fish (Grabher & Wittbrodt, 2008; Sano *et al.*, 2009; Grabher *et al.*, 2003; Thermes *et al.*, 2002). Transgenesis aided by meganuclease I-SceI, which recognize 18-bp sequences flanking the DNA construct, can lead to approximately 30% of GFP positive F0 zebrafish to produce transgenic offsprings (Thermes *et al.*, 2002). Similarly, reconstructed *Sleeping beauty* transposon system can achieve about 31% of germline transmission rate (Grabher *et al.*, 2003). Another reconstructed transposon system, *Frog prince* (Miskey *et al.*, 2003), also resulted in a good rate of germline transmission in medaka (Sano *et al.*, 2009). *Tol2*, a transposon originated from medaka (Koga & Hori, 2001), has been a popular tool in generation of transgenic zebrafish, achieving high germline transmission rate (Parinov *et al.*, 2004; Kawakami, 2007) (Kawakami *et al.*, 2004). Unfortunately, *Tol2* is not suitable for medaka transgenesis despite the high successful rate in zebrafish. Low transpositional activity of *Tol2* has been reported in Philippines medaka (*Oryzias luzonensis*) whose genome does not contain *Tol2* (Koga *et al.*, 2002). Regardless of the low transpositional activity, *Tol2* is actively functional in natural populations of *O. latipes* as indicated by southern blot (Koga & Hori, 2001). Hence, endogenous *Tol2* activity may lead to redistribution of *Tol2* transgene in future generations in medaka.

Ac/Ds, a transposon that is derived from maize, is a member of large hAT family of "cut and paste" transposons (McClintock, 1951). Activator, *Ac,* is autonomous element which encodes a transposase between *cis* terminal repeats. Dissociation, *Ds,* contains only terminal repeats which can only be *trans* activated in the presence of *Ac* transposase. This Ac/Ds system has been popularly used in the transgenesis of various plant species (Izawa *et al.*, 1991; McClintock, 1951; Bancroft *et al.*, 1992). Although maize Ac/Ds element is the first DNA transposon discovered, it has been only recently shown to be functional in different kingdoms such as yeast, zebrafish and mammalian cell culture (Weil & Kunze, 2000; Emelyanov *et al.*, 2006). This indicates the non-requirement of host specific factors for its function. In zebrafish, about 57% of the F0 fish screened have been confirmed for germline transmission of microinjected DNA, demonstrating the high efficiency of the Ac/Ds system in fish transgenesis (Emelyanov *et al.*, 2006). At the initiation of the project, there was no reported literature about using Ac/Ds transposon in medaka model. Thus, we first tested the validity of using Ac/Ds transposon for establishing stable transgenic medaka

4.2.1 Efficient germ-line transmission of transgene in medaka by using Ac/Ds transposon

We first evaluated the efficiency of Ac transposase with transient transgenic assays by injecting plasmid pDsKRT4-GFP or pDsHSP70-GFP and demonstrated that GFP expression was increased by co-injection with *Ac* mRNA (Fig. 3.9 & 3.10) which is likely due to early integration of transgene during embryonic development (Grabher & Wittbrodt, 2008). Thus Ac transposase improves the rate of integration of transgene.

Furthermore, high efficiency of germline transmission rate is observed in the two transgenic lines, Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*) (Table 3.1). Out of 16 GFP-expressing founder fish screened [12 for Tg(*hsp70:gfp*) and 4 for Tg(*cyp1a:gfp*)], a total of 14 F0 founders showed germline transmission and thus the successful rate is 87.5% in GFP-expressing founders or 74% if non-GFP positive founders is considered too. Typically <10% of germline transmission rates have been reported using conventional microinjection of naked DNA plasmid (Miyamoto *et al.*, 2009; Kinoshita *et al.*, 2000; Zeng *et al.*, 2005; Thermes *et al.*, 2002) Our results also compare favorably with two other common transgenic approaches in medaka: 30% with meganuclease I-SceI(Thermes *et al.*, 2002) and 31% with sleeping beauty(Grabher *et al.*, 2003). Concurrently, another group has also reported the ease of using Ac/Ds system in the generation of transgenic medaka which they have achieved about 30% germline transmission rate (Froschauer *et al.*, 2012).

The frequencies of transgenic F1 progeny in Tg(*hsp70:gfp*) range from 2.9% to 100% while that in Tg(*cyp1a:gfp*) range from 24.4% to 88.2% (Table 3.1). The statistics of Tg(*cyp1a:gfp*) may be underestimated as we have only screened for constitutive GFP expression but not induced GFP expression. Moreover, since visual screening was performed for the transgenic frequency, we might miss the transgenic individuals that did not express the transgene due to chromosome effects, silencing effects and other reasons. PCR determinations may improve the transgenic rate further. Nevertheless, these observations indicate that the Ac/Ds maize transposon is highly efficient, thus greatly reducing the time and effort spent on screening for germline transmission.

4.2.2 Typical transition of Ac/Ds is retained in transgenic medaka

In transgenic zebrafish generated using the Ac/Ds transposon system, the average insertions in F0 founder is four (Emelyanov *et al.*, 2006). Another study which used the Ac/Ds transposon system in medaka model has identified up to five integrations in individual fish (Froschauer *et al.*, 2012). In the present study, Southern blot of the two transgenic lines also shows multiple insertion: four different insertions in Tg(*hsp70:gfp*) F0 family and 3 insertions in Tg(*cyp1a:gfp*) F1 family (Fig. 3.13). It is interesting to note that the offsprings inherited different transgenic insertions, indicating that there is transgenic variation in the germ cells.

Furthermore, we isolated the genomic DNA fragments flanking the transgene from four selected transgenic lines which have been verified to have single insertion site based on Southern blot analysis (Fig. 3.14). Sequences flanking the *minDs* elements from all these transgenic lines, except for Tg(*cyp1a:gfp*) 4.2, revealed classical 8-bp direct duplication of the genomic insertions (Fig. 3.15). 8-bp direct duplication of target site is also previously described in both transgenic plants and transgenic zebrafish generated by using the Ac/Ds transposon (Izawa *et al.*, 1991; Emelyanov *et al.*, 2006; Weil & Kunze, 2000). However, the flanking sequence of Tg(*cyp1a:gfp*) 4.2 was an exception and seemed to have an anomaly in its 8-bp target duplication. The red bold underlined sequence in Fig. 3.15 appeared to be reminiscent of DNA hairpin intermediate seen in DS excision in yeast (Weil & Kunze, 2000). The mechanism of Ac/Ds transposition is not exactly known (Gorbunova & Levy, 2000) but circumstantial evidence highly favors the excision mechanism towards hairpin model proposed by Coen 1989 et al (Coen *et al.*, 1989). Perhaps the aberrant sequence is the result of abortive excision of inserted transgene by the transposase that was translated from injected *Ac* mRNA during microinjection. In all the analysis of Ac/Ds mediated transgenic lines, no plasmid backbone or concatemers were detected besides

minDs elements in the flanking sequences, indicating clean and single transpositions that are similarly observed from the *Ac* transposition in zebrafish (Emelyanov *et al.*, 2006).

4.2.3 Genomic analysis of integration sites.

The F1 offsprings of Tg(*hsp70:gfp*) 1.1, Tg(*cyp1a:gfp*) 1.1, Tg(*cyp1a:gfp*) 3.2 and Tg(*cyp1a:gfp*) 4.2 all showed a single insertion based on Southern blot analysis (Fig. 3.14) which is confirmed by their Mendelian inheritance ratio from their outcross with wild type fish (Table 3.2). The flanking sequences of the transgenes were searched by BLAST against the genome database in Ensembl Browser to determine their genomic location (Fig.3.16-3.19). Interestingly, the putative insertion site in the BLAST results of Tg(*cyp1a:gfp*) 1.1 and Tg(*cyp1a:gfp*) 4.2 do not show 100% identity to their extracted flanking sequences even though the genetic background of the transgenic fish is also of the same lab strain (Hd-Rr) for genome sequencing. This could be due to single nucleotide polymorphisms or repetitive regions in the genome that complicate the BLAST analysis against the genome database. Only BLAST result sequences from Tg(*cyp1a:gfp*) 3.2 and Tg(*hsp70:gfp*) 1.1 has more than 95% identity to its flanking sequence. However, the BLAST result of Tg(*hsp70:gfp*) 1.1 only reveals the location in contiq sequence which has yet to be mapped to the chromosome. Future updates of the genome data would help to pinpoint the insertion of transgene clearly and PCR, with primers annealing to the flanking sequence, can be performed to affirm the insertion. As such, so far PCR has confirmed that the transgene insertion of Tg(*cyp1a:gfp*) 3.2 is in chromosome 3 (data not shown). Nevertheless, the BLAST results reveal that transgene insertion for all lines are not in gene coding region except for Tg(*hsp70:gfp*) 1.1. The insertion of Tg(*hsp70:gfp*) is shown to be in the intronic regions of two novel genes. However, we

did not observe any abnormal growth nor behavior in this Tg(*hsp70:gfp*) line and also the other lines, suggesting that the transgene insertion did not cause major disruption to nearby genes. Tg(*hsp70:gfp*) 1.1 also did not show a particular constitutive GFP expression, hence implying that the transgene was not greatly affected by the transcriptional regulation of the unknown genes. Preliminary results (Fig. 3.21, 3.22, 3.24, 3.26) have shown that all our selected transgenic lines tested were able to respond to respective inducers regardless of their insertion loci.

4.2.4 The potential of Ac/Ds transposon system in gene/enhancer trap in medaka

From the present study, a plethora of GFP expression patterns were observed in the F1 generation of Tg(*cyp1a:gfp*) lines (Fig. 3.14) and many of the GFP-expressing tissues such as brain and notochord are not known to express *cyp1a*, signifying that GFP expression was most likely influenced by a nearby tissue specific enhancer. Such observations indicate the potential of using this transposon to generate gene trap and enhancer trap in medaka. In zebrafish model, a few large scale enhancer traps and gene traps have been generated using *Tol2* and *Sleeping beauty*. While in medaka, small scale enhancer trap and gene trap studies have been reported using reconstituted transposon system, *Sleeping beauty* (Grabher *et al.*, 2003) and *Frog prince* (Sano *et al.*, 2009). In the enhancer trap study that utilized *Sleeping beauty* transposon, 21 novel GFP expression pattern were generated but complete or partial plasmid insertion and transgene concatemers have been detected in many of them (Grabher *et al.*, 2003), hence complicating the GFP reporter signal. Recently, gene trap system using Ac/Ds in medaka were generated (Froschauer *et al.*, 2012) and 15 different GFP phenotypes were identified and established. The presence of *Ds* element made it easier to locate genomic integration site and subsequently the gene that was disrupted due to insertion of transposon.

Due to space constraint and limited resources and time, the variation in GFP patterns observed in Tg(*cyp1a:gfp*) (Fig. 3.12) is not further investigated although it seemed that transgene *cyp1a* promoter behaved like minimal promoter in an enhancer trap system. Nevertheless, it is possible to generate an enhancer trap system again by reinjecting *Ac* transposes mRNA alone in the embryos of transgenic Tg(*cyp1a:gfp*). Previously, it has been demonstrated that exisitng transgene flanked with *minDS* elements was translocated again to a new genomic site when injected with *Ac* mRNA during one-cell embryo stage of a stable transgenic zebrafish (Emelyanov *et al.*, 2006).

Induced responses of the three single-insertion Tg*(cyp1a:gfp)* lines were similar but Tg*(cyp1a:gfp)* 1.1 expressed in lesser extent than the other two lines (Fig.3.26). Also, no GFP expression was observed in the mast cells of Tg*(cyp1a:gfp)* 1.1 at 6 dpf but it was observed in Tg*(cyp1a:gfp)* 3.2 and Tg*(cyp1a:gfp)* 4.2 after five days of exposure in 5 nM of TCDD (data not shown). Position effects and epigenetic regulation could account for the differences in their induction phenotype. In epigenetic studies using transgenic zebrafish, the reduction in transgene expression phenotypes observed in the next generation was shown to be associated with increased DNA methylation of the transgene and certain tissues/region seemed to be prone to silencing (Goll *et al*, 2009). Transgene silencing tends to occur in transgenic fish that carry concatemers of injected plasmid DNA (Kawakami, 2005) or repeat regions such as those in GAL4-UAS (Goll *et al*, 2009). As Ac/Ds-aided transgenesis of fish was fairly recent, there is no hitherto report of transgene silencing observed in transgenic fish using this system though there were a few reports of transgene silencing in plant using Ac/Ds (Izawa et al. 1997, Kim et al, 2002). Nevertheless, bisulphite sequencing could be performed to determine the methylation status of Tg*(cyp1a:gfp)* medaka.

4.3 Chemical exposure of Tg(*hsp70:gfp*)

4.3.1 Differential induction of GFP expression in different tissues by different heavy metals

Mercury, arsenic and cadmium are some of the heavy metals in the list of priority pollutants by EPA (United States Environmental Protection Agency, 1981). In the present study, we have treated Tg(*hsp70:gfp*) fry with these heavy metal salt solutions at various concentrations for 24 hours to examine their induced GFP expression. Generally, all three heavy metal salt solutions induced GFP expression in fry and the common inducible organ of Tg(*hsp70:gfp*) medaka in all the heavy metal treatments is the liver. Heat shock induced ubiquitous GFP expression in the transgenic medaka (Fig. 3.24G), implies that most, if not all, cells are capable of inducing GFP expression under the *hsp70* promoter. However, there are differences observed in the spatial GFP expression between the heavy metal treatments. For example, GFP was induced in muscle only in arsenic salt treatment (Fig. 3.30) while it was induced in olfactory pits only in cadmium chloride treatment (Fig. 3.29). Induced GFP expression in nervous system was observed only in mercury chloride treatment (Fig. 3.28). These observations suggest that different organs/tissues may have different sensitivity of induction threshold by different metals and/or that different metals may be bioaccumulate preferentially in different organs/tissues. Numerous studies have demonstrated that the rate of accumulation of the same metal differs in various organs of fish and the order of accumulation in various organs is different for various metals under the same condition (Huang *et al.*, 2007; Jabeen & Chaudhry, 2010; Ebrahimi & Taherianfard, 2010; Jarić *et al.*, 2011). To determine whether the accumulation of metals in various organ correlates to transgenic GFP expression or *hsp70* upregulation, further analysis can be done by investigating the

concentration of metals in various tissue harvested from treated fish. Mercury level is best determined by cold vapor atomic fluorescence spectroscopy due to its volatile nature while other metal level can be determined by inductively coupled plasma emission spectrometry (Subramanian, 1996).

4.3.2 Transgenic GFP expression in mercury treatment

Mercury exists in three forms: elemental mercury known as metallic mercury, inorganic mercury compounds such as mercurous or mercuric salt, and organic mercury, primarily methylmercury. The toxicity profiles of the three forms of mercury are likely to be different. For example, methylmercury has major toxic effects on nervous system while inorganic mercury can cause renal tubular dysfunction as well as immunotoxic response (Guzzi & La Porta, 2008). However, conversion to other forms of mercury can occur after uptake by the organism. It is thought that microorganisms present in the sediments of water bodies can convert inorganic mercury via biomethylation to methylmercury. Methylmercury then enters into aquatic food chain when fish consume these microorganisms. In aquatic pollution, dominant forms of mercury present can be inorganic mercury from industrial waste dumping or methylmercury through consumption of contaminated fish and other seafoods.

We used mercury chloride salt solution to treat Tg(*hsp70:gfp*) medaka. GFP expression was observed weakly in kidney and gills at the lowest concentration used (200 µg/l) (Fig 3.27). As the concentration of mercury chloride increased to 1000 µg/l, other organs such as liver, skin and notochord also showed GFP expression. GFP expression was detected in transgenic fry at sublethal concentration as low as 200 µg/l of mercury chloride and all fries expressed GFP at 800 µg/l of mercury chloride. It has been previously shown that mercury accumulated more in gills and kidneys

compared to liver and muscle within the same exposure period in the carp (Ebrahimi & Taherianfard, 2010), thus consistent with our observations about the expression of GFP in kidney and gills probably due to the preferential accumulation of mercury in these organs at low concentration. A new imaging technique using synchrotron x-ray fluorescence has been developed to investigate bioaccumulation of methylmercury in zebrafish larvae in real time (Korbas *et al.*, 2008). Methylmercury cysteine, a common chemical form of mercury exist physiologically (Harris *et al.*, 2003), has been found to be most concentrated in the lens of zebrafish. Methylmercury cysteine also accumulated significantly in organs such as brain, liver, muscle, gut, kidney tubules and pectoral fins. Other studies report significant levels of mercury in brain, liver, kidney and skeletal muscles in fish after mercury exposure (Gonzalez *et al.*, 2005; Branco *et al.*, 2011). Therefore, our observations of GFP expression in notochord, liver, kidney, gills and skin of Tg(*hsp70:gfp*) medaka in mercury treatment is quite consistent with the above literatures.

4.3.3 Transgenic GFP expression in cadmium treatment

Common health effects due to exposure to cadmium are renal dysfunction and also bone diseases such as itai-itai disease in which symptoms like bone fractures, pseudofractures, deformed spines and severe generalized pain due to osteomalacia. International agency for research on cancer (IARC,1993) has classified cadmium as a human carcinogen (group 1) based on both experimental evidence from animal studies and human epidemiological studies. Furthermore, cadmium has been suspected to be a potent metallo-estrogen as estrogenic responses were observed in *in vivo* studies after cadmium exposures. One of the major sources of cadmium exposure is through dietary intake such as consumption of crops that has high level of cadmium uptake from contaminated soils or consumption of contaminated drinking water. One

famous example of cadmium pollution is the dumping of cadmium waste into Jinzu River from mining companies in Toyama Prefecture, Japan around 1912. As the river was used for irrigation of rice field, drinking and fishing activities, cadmium poisoning was observed in mass populations in that prefecture (Horiguchi *et al.*, 2010).

In our cadmium chloride treatment of Tg(*hsp70:gfp*) medaka, GFP expression was first detected in organs such as the kidneys at the lowest concentration used (25 μg/l), followed by liver and olfactory pits and skin as the cadmium chloride dosage increased (Fig. 3.28). In another similar study using transgenic *hsp70*-eGFP zebrafish,GFP expression is first detected in the gills, skin and olfactory organ, followed by liver and pronephric ducts at the highest concentration used, 125 μM (23 mg/l) of cadmium chloride (Blechinger *et al.*, 2002). Tg(*hsp70:gfp*) medaka expressed GFP in lesser organs/tissues and in different order of sensitivity as compared to that in *hsp70*-eGFP zebrafish, in cadmium treatment. The differences observed between these two transgenic fish species maybe due to different factors. 3 hour exposure was performed in the *hsp70*-eGFP zebrafish study while our study used continuous exposure for 24 hours. Other than that, it maybe due to species differences in their sensitivity towards chemical and their responses. Nevertheless, the sensitivity of the two heat shock transgenic lines in two different species is comparable since lowest concentration to observe GFP expression in *hsp70*-eGFP zebrafish line is at 0.2 μM (36.6 μg/l) while that in Tg(*hsp70:gfp)* medaka is at 25.5 μg/l .

4.3.4 Transgenic GFP expression in arsenic treatment

Similar to mercury, arsenic can exist in various forms such as organic species, elemental form and inorganic forms including trivalent arsenite or pentavalent arsenate. Generally, elemental form of arsenic is considered as non poisonous, however, various metalloid arsenic compounds has different toxicity (Luh *et al.*,

1973). The toxicity of the various forms of arsenic in decreasing order is as follows: Arsenite (As^{III})> Arsenate (As^{V})> organic species of arsenic. Exposure of arsenic can lead to renal disease, liver disease, neurological disorders, gastrointestinal irritation, cardiovascular dysfunctions and cancers, particularly skin cancer (Jomova *et al.*, 2011). In water, arsenic is normally present as As^{V} but will be biotransformed to arsenite after consumption by humans or other organism (Ventura-Lima *et al.*, 2011). In our study, sodium arsenate exposure has induced GFP expression in organs in the following order according to its sensitivity: liver, gut, muscle and skin (Fig. 3.29). The lowest concentration required to induce GFP expression is between 12.5 µg/l to 25 µg/l. In a transient assay using microinjected zebrafish embryos with human *HSP70* promoter linked to GFP gene, GFP expression was detected in some cells of gills, skin, olfactory epithelium cells, neuronal cells and myotubes at 50 µm (6.3 mg/l) and above of arsenite salt solution (Seok *et al.*, 2007). Our observations of Tg(*hsp70:gfp*) medaka appears to be consistent with that of transient transgenic *hsp70* zebrafish, except that our fish represent a stable line induction and also higher sensitivity (12.5-25 µg/l) towards arsenic.

4.3.5 Other chemical treatments

Other than heavy metals, we also exposed the Tg(*hsp70:gfp*) fish to other types of pollutants such as 4-nitrophenol, bisphenol A,TCDD and lindane (Fig. 3.30-3.33). Except for lindane treatment, no GFP expression was induced even at lethal dosage after two days of treatment. The GFP expression observed in lindane treatment is relatively weak as compared to the heavy metal treatments, with GFP expression only in discrete bundles of muscle fibers at concentrations of 5 mg/l or higher (Fig. 3.33). Deformities observed in lindane-treated fry include crooked and shrunken body

with swimming difficulties, which implies that muscles were significantly damaged (Table 3.3).

In mammalian model, the major effect of lindane is neurotoxicity due to its lipophilic nature and other serious effects that include degeneration of cardiac muscle, necrosis of blood vessels and liver (Nolan *et al.*, 2012). Exposure to lindane during early development of fish, such as gilthead seabream (*Sparus aurata*) fry, has revealed myoskeletal defects, weak swimming, trembling, skin opacity, depigmentation and exophthalmia (Oliva *et al.*, 2008). Zebrafish embryos show reduced growth when exposed to 40 μg/l of lindane in early stage (Görge & Nagel, 1990). Thus, in aquatic organisms, lindane most likely causes detrimental effects on muscle. Muscle damages caused by lindane perhaps induce stress in individual muscle fibers which may lead to up-regulation of the stress marker gene, *hsp70,* and also GFP expression in Tg(*hsp70:gfp*) fry.

4.3.6 GFP induction in adult Tg(*hsp70:gfp*) medaka

Induction of GFP expression was also tested in six-month-old fish using mercury (Fig. 3.34). The only externally detectable GFP expression in live fish is in brain region for this treatment. GFP expression took more than 24 hours to be visible externally in 400 ug/l mercury chloride treatment as no GFP expression was detected in the live transgenic fish in any treatment group after 24 hours of treatment. For lower concentration (200 ug/l mercury chloride), it took about four or five days of exposure to observe GFP expression externally. It is possible that if the exposure condition is extended beyond five days of treatment, GFP may be detected in 100 ug/l mercury chloride treated group. During dissection after five days of treatment, GFP expression was observed weakly in the kidney, moderately in the gut at all mercury chloride-treated group while GFP expression was patchy in liver only in the 400 μg/l

group. This observation is in contrast with the results from mercury treatment of larvae where the liver and kidney appeared to be the most sensitive organs to induce GFP upon mercury treatment. The liver at adult stage is likely to be more tolerant of stress than it is at larvae stage. Nevertheless, GFP expression induced by mercury chloride is sufficient for external observation without the need for dissection by 72 hours of exposure.

4.3.7 Conclusion on Tg(*hsp70:gfp*)

Tg(*hsp70:gfp*) responded well towards heavy metal as mercury, arsenic and cadmium hence it may also be responsive to other heavy metals such as lead, chromium and nickel. Interestingly, although HSP70 is known to be a general stressor protein, Tg(*hsp70:gfp*) had not induced GFP expression significantly towards other types of pollutants such as BPA, TCDD, 4-nitrophenol and lindane even at lethal concentration. Possibly, the presence of metals could activate a signalling pathway that crosstalks with HSF-1 signaling or directly activates *hsp70* promoter. The Tg(*hsp70:gfp*) medaka fry is able to induce GFP expression at sublethal concentration of the heavy metals within 24 hours, indicating the possibility of quick detection of pollutants using transgenic line. Some heavy metals, such as mercury and arsenic, exist in few forms (inorganic/organic or variation in valence form) which give rise to different toxicity profiles. Hence, it is uncertain whether the transgene expression remains the same when different form of metal is used instead.

4.4 Chemical exposure of Tg(*cyp1a:gfp*)

4.4.1 GFP expression was observed in similar organs in both PAHs and dioxin treatments

PAHs and dioxin are persistant organic pollutants (POPs) which are resistant to environmental degradation and hence can persist in soil and sediments for periods extending from decades to centuries. They can enter the aquatic ecosystem through effluent, atmospheric deposition, petroleum spill, run-off and ground water. Xenobiotic degradation of PAHs and dioxins is commonly thought to be mediated via AhR pathway in cells. Activation of AhR pathway up-regulates genes such as *cyp1a* which catalyze the oxygenation of PAHs and heterocyclic aromatic amines/amides, the demethylation of aminoazo dyes, and the dealkylation of phenacetin, caffeine and other agents (Ma & Lu, 2007). To assess the response of Tg(*cyp1a:gfp*) medaka, the fish were exposed to three chemicals that have been shown to upregulate *cyp1a* gene-TCDD, BAP and 3-MC. Both BAP and 3-MC are polycyclic aromatic hydrocarbons while TCDD belongs to a group of chlorinated organic chemicals termed as dioxin. Acute exposure to high level of dioxin can cause liver damage and chloracne (a chronic inflammatory skin condition characterized by keratinous plugs with cysts and dark acnes) while chronic exposure to dioxin is often associated with detrimental effects in nervous, immune, reproductive, and endocrine system (Marinković *et al.*, 2010). Chronic exposure to PAH is known to cause carcinogenic as well as mutagenic effects (Srogi, 2007) and emerging evidence indicates that PAH exposure can also affect the immune system (Carlson *et al.*, 2004).

Based on our experiments with the three compounds, it appears that the common organs to express GFP in Tg(*cyp1a:gfp*) include the liver, kidney, gut and tail fins (Fig. 3.35-3.37). Generally, GFP expression was the most intense in the liver compared to the rest of the organs in the three compound exposures. It is not surprising as *cyp1a* up-regulation is often observed in organs that are involved in xenobiotic pathways including the liver, kidney, gut (Sarasquete & Segner, 2000).

However, in TCDD exposure, we subsequently observed GFP expression in other organs such as mast cells, gills, olfactory pits, blood vessels and strongly in tail fin in higher concentrations of TCDD. The concentration of BAP and 3-MC used in this study may not be sufficient to obtain GFP expression in these organs as observed in TCDD exposure. TCDD apparently induces the highest and strongest reaction of *Tg(cyp1a1:gfp) 4.2* at a concentration (0.322 µg/l) which is much lower than BAP (62.5µg/l) and 3-MC (6.25 µg/l), signifying that the Tg(*cyp1a:gfp*) is very sensitive to TCDD. TCDD is known to be the most toxic cogner of dioxin and one of the strongest agonist of AhR. Furthermore, its halogenated aromatic structure makes it less susceptible to biotransformation which results in its long elimination half life (Grimwood & Dobbs, 1995) while PAHs are generally less persistent (Hahn, 2002). Hence, Tg(*cyp1a:gfp*) responds strongly towards TCDD even at low concentrations. Since GFP expression is consistent with in situ hybridization of endogenous *cyp1a* transcript (Fig. 3.38), GFP expression observed in other organs indicates that the organs are capable of *cyp1a* induction but perhaps only due to high dosage or toxicity of the tested compounds.

4.4.2 Transgenic GFP expression in 3-MC treatment

3-MC is artificially synthesized and commonly used as cancer inducing agent in experimental studies. There are fewer studies investigating the exposure to 3-MC in fish as compared to mammals. In Tg(*cyp1a:gfp*) fry treated with 3-MC, GFP expression was mainly induced in the liver, kidney, gut and , at high dosage (100 µg/l), in the tail fin (Fig. 3.35). The lowest concentration of 3-MC required to induce GFP expression in fry is at 6.25 µg/l, a concentration that does not cause major pathology in the fish. Thus, it signify that the transgenic line is rather sensitive to 3-MC.

4.4.3 Transgenic GFP expression in BAP treatment

Similar to 3-MC treatment, GFP induction in BAP-treated Tg(*cyp1a:gfp*) fry is mainly observed in the liver, kidney, gut and ,at high dosage (1000 μg/l), in the tail fin (Fig. 3.36). Apparently, GFP is often induced in liver, kidney and gut simultaneously in most of the treated fry. The sensitivity of Tg(*cyp1a:gfp*) is around 62.5 μg/l or even lower as the lowest concentration we tested has induced GFP expression in majority (>80%) of the fry. Since no distinct abnormal morphology or mortality was observed during BAP treatment even at the highest concentration, the detection of BAP is more reliable using GFP induction of Tg(*cyp1a:gfp*) than to base on the morphology of the fry. To visualise the distribution of BAP or its metabolites in BAP exposed medaka embyo, multiphoton laser scanning microscopy (MPLSM) was used to detect the fluorescence emitted from BAP and BAP metabolites in medaka embryo (Hornung *et al.*, 2007). Fluorescence detected by MPLSM was limited to yolk, gall bladder, gastrointestinal tract and biliary system after BAP treatment of the fry, signifying that the BAP as well as its metabolites were likely to be present in these organs. This, together with our observations, suggests that the gut may also play an important role for metabolizing the BAP besides liver and kidney.

4.4.4 Transgenic GFP expression in TCDD treatment

The liver and kidney were the most sensitive organs to express GFP in the presence of TCDD at 3.22 ng/l (0.01 nM) (Fig 3.37). Thus, the sensitivitiy of Tg(*cyp1a:gfp*) towards TCDD is comparable to EROD assay from cell culture which has detection limit from 0.77 ng/l to 322 ng/l (2.4 pm to 1 nm) (Zhou *et al.*, 2006; Sanderson *et al.*, 1996). Similar transgenic cell cultures using dioxin responsive elements have been developed and have achieved sensitivity in pM range of TCDD(Elskens *et al.*, 2011; Sanderson *et al.*, 1996). However, our transgenic medaka

is the only in vivo vertebrate model that is capable of detecting TCDD and other PAHs, in a true physiological context.

Besides liver and kidney, other organs that express GFP include the gut, mast cells, gills, tail fins, olfactory pits and sometimes blood vessels at higher concentrations of TCDD exposure. Note that mast cells, olfactory pits, gills and skins were organs that were in direct exposure with toxicants. This implies that *cyp1a* upregulation were induced in these organs, however at a higher TCDD concentration. *In situ* hybridization with *cyp1a* mRNA probe further affirmed the induction of *cyp1a* in these organs (Fig. 3.25). In zebrafish embryos exposure to TCDD, *cyp1a* was induced, either at protein or transcript level, in skin, vasculature, kidney, gastrointestinal, liver as well as heart (Yamazaki *et al.*, 2002). Likewise, in other teleostean fishes including gilthead bream, mummichog and rainbow trout etc., shown similar tissue distributions of induced *cyp1a* mRNA as mentioned above and also in organs such as gall bladder, gills, gonads, nervous tissue and endocrine cells (Sarasquete & Segner, 2000). Perhaps due to differences in species or inducers used, we did not observe *cyp1a* expression in some organs such as gall bladder, heart and nervous tissue in our TCDD-treated transgenic medaka. To our knowledge, this is the first report demonstrating *cyp1a* gene activity in mast cells of the lateral line of the fish when exposed to high dosage of TCDD.

Generally, with increased concentrations of TCDD, more organs will express GFP. The order of key organs that expressed GFP from low concentration to high concentration of TCDD is as follows: liver, kidney, gut, olfactory pits, tail fin, gills and mast cells (Fig. 3.37). Although the GFP expression is difficult to quantify and extrapolate to the TCDD concentrations, the order of organs that express GFP can be an rough indicator of relative concentrations of TCDD.

4.4.5 Other categories of pollutants

Besides treating the Tg(*cyp1a:gfp*) with POPs, we also exposed the fish to other types of pollutants such as 4-nitrophenol, bisphenol A, mercury and lindane (Fig. 3.38-3.41). Except for 4-nitrophenol, no GFP expression is induced even at a lethal dosage after two days of treatment. Although GFP expression is induced in 4-nitrophenol treatment, expression is weak and patchy in the liver in small percentage (17.2%-20.7%) of the fry and only at the concentration above 7.5 mg/l (Fig. 3.41). The major metabolic route of 4-nitrophenol, which accounts for approximately 70% of the dose, is via conjugation to form either glucuronide or sulphate conjugates. However, saturation kinetics may be reached when 4-nitrophenol concentration is increased further. This may lead to a increase in other metabolism pathway of 4-nitrophenol such as oxidation to 4-nitrocatechol or reduction to 4-aminophenol (ATSDR, 1992). Increase in these side metabolites may therefore leads to up-regulation of *cyp1a* to degrade such metabolites, resulting in weak GFP expression in some of the hepatocytes as observed at high concentrations. Due to the insignificant number of positive results, it is unlikely that Tg(*cyp1a:gfp*) is a reliable indicator for the presence of 4-nitrophenols.

4.4.6 GFP induction in adult Tg(*cyp1a:gfp*) medaka

We performed TCDD treatment, with concentrations from 32.2 ng/l (0.1nM) to 805 ng/l (2.5 nM) on six-months-old adult Tg(*cyp1a:gfp*) to investigate the induction of GFP expression externally (Fig. 3.42). After one day of treatment, GFP expression was observed intensely in the kidney region, urinary pore and olfactory pits in all TCDD treated fish externally. After three days of treatment, GFP expression was further intensified in these organs as well as in other organs such as skin, gills etc. Dissection of the TCDD-treated fish has also shown that the liver and the gut

expressed GFP intensely. Due to the peritoneal abdomen membrane of the fish, GFP expression in the liver and the gut was not so obvious by external observation. The expression pattern we observed in adult fish is similar to the pattern observed in larvae, demonstrating the consistent toxicology profiles of TCDD in both developmental stages. As GFP is easily detected from the kidney region externally in about 24 hours time, this transgenic line can be an effective and sensitive detector of TCDD.

4.4.7 Conclusions on Tg(*cyp1a:gfp*)

Tg(*cyp1a:gfp*) was generally able to respond to the two PAHs and dioxin by expressing GFP in liver, kidney and gut. Detection of GFP expression within 24 hours at low concentration where no obvious morphological changes were observed, demonstrates the sensitivity and rapidness of Tg(*cyp1a:gfp*) as biomonitoring tools. Tg(*cyp1a:gfp*) did not respond to other categories of pollutant such as bisphenol A, mercury, lindane and 4-nitrophenol. Thus this transgenic line can be highly specific to certain categories of POPs such as PAHs and dioxins, and can be a valuable biomonitoring tool for these categories of pollutants.

4.5 Summary

In summary, we have demonstrated that maize Ac/Ds system is highly efficient in germline transmission of microinjected DNA in transgenic medaka and typical features of Ac/Ds transposition exist in the medaka model, including 8-bp direct duplication target site and multiple insertion sites. Furthermore, the high germline transmission and easily detectable genomic integration sites make the Ac/Ds system an attractive tool to generate gene trap or enhancer trap in medaka.

Using the Ac/Ds system, we have also established the two types of transgenic medaka lines, Tg(*hsp70:gfp*) and Tg(*cyp1a:gfp*), for environmental monitoring and characterized their responses to various chemical exposures. We found that their detection limits are sufficient to induce GFP expression below sublethal concentration of many chemicals. We have also demonstrated the potential of using both Tg(*hsp70:gfp*) and Tg*(cyp1a:gfp)* adult fish for biomonitoring.

4.5.1 Limitations and improvements

The transgenic biomonitoring fish system has its own limitations or disadvantages. The first major disadvantage is that transgenic fish can not predict the exact chemical as compared to chemical analytical approaches. Generally, the design of transgenic fish is such that it is a broad based sensor which responds to a group of chemicals with similar structure or properties. However, if certain chemical exposure induces a specific GFP spatial expression in the transgenic fish, it is possible to deduce the suspected contaminant based on the transgenic expression. As such, Tg(*hsp70:gfp*) seems to show metal-specific GFP induction for the three heavy metals tested, arsenic, mercury and cadmium. However, more exposures with other heavy metals, e.g. chromium, lead and copper, has to be performed on Tg*(hsp70:gfp)* 1.1 to ascertain the specificity of transgene expression.

Secondly, the sensitivity of the transgenic fish is generally lower than current chemical analytical approaches (US Environmental Protection Agency, 2011). Both transgenic lines developed in the present study are unlikely to detect concentration as low as the maximum contaminant level (MCL) of EPA for drinking water standard (US Environmental Protection Agency, 2009) within 24 hours of exposure, though we have yet to determine LOEC for some of the chemicals. MCL values of most chemicals (Table 3.4 & Table 3.5), except for that of sodium arsenate, is at least 10

fold lower than the lowest concentration used in this study in which faint GFP was induced. Perhaps longer exposure of chemical present in low concentration is required so that the transgenic fish can bioaccumulate sufficient amount to activate transgene expression. Possibly, the usage of homozygous transgenic fish instead of hemizygous fish could further increase its sensitivity.

Induction of gene expression can be influenced by other factors which may directly affect the gene expression and/or may act indirectly by crosstalking with other transcription factors that control the gene expression. *cyp1a* expression was reported to be affected by hormonal factors such as glucocorticoids, insulin and sex hormones (Monostory *et al.*, 2009). In transfected HepG2 cells, addition of dexamethasone, an agonist of glucocorticoid receptor, repressed TCDD-mediated induction of AhR transcriptional activity (Dvorak *et al.*, 2008). Dexamethasone treatment also inhibited stress-induced HSF1-mediated chloramphenical acetyltransferase reporter gene expression in the stably-transfected mouse L929 cell (Wadekar *et al.,* 2001), suggesting that HSF signaling pathway crosstalk with glucocorticoid receptor pathway. Often, more than one chemicals exist in the water. Hence, it will be of interest to expose the transgenic lines to combinations of various chemical to determine whether their reporter gene expression will be synergetic, repressive or additive phenomenon under respective chemical induction.

One major limitation of this study is the lack of quantitative analysis of GFP expression. As such, it was uncertain that the intensity of induced GFP expression in transgenic fish could correlate to the concentration of the contaminant exposure. Karauchi *et al.*, 2005 developed a method to quantify the amount of GFP in the liver and has shown that GFP expression induced in the liver of transgenic fish was concentration dependent. However, as demonstrated in both Tg*(cyp1a:gfp)* and

Tg*(hsp70:gfp)* exposure, GFP expression often occurs in more than one organ. Furthermore, some chemicals induce GFP in several organs and in different order of induction. For example, as TCDD exposure increased up to 322 ng/l , more organs such as mast cells, gills and olfactory pits started to express GFP while the intensity of GFP in three main organs- liver, kidney and gut appeared to be saturated after 32.2 ng/l of TCDD. Thus it was difficult to determine the concentration based on the intensity of the GFP in single selected organ. For each chemical exposure, it was necessary to optimize the analysis to determine a linear relationship between GFP expression and the chemical exposure.

4.5.2 Future directions

Fish embryos are popularly used for toxicology tests as they are not regulated by current legislations on animal welfare in Europe (Scholz *et al.*, 2008). The fecundity of the medaka is not as good as that of zebrafish as the medaka produces about 20 embryos each time while zebrafish can produce up to 300 embryos each time. Thus using zebrafish instead of medaka for embryo toxicology test would be more advantageous since it is possible to carry out high throughput screening with the availability of a large number of embryos.

Nevertheless, medaka fares better than zebrafish in terms of adaptability and tolerance hence medaka is more suitable for on site biomonitoring adult fish. From the adult exposure, we noticed that the observable GFP expressions in live fish were from organs that were close to the body wall such as brain and kidney. Breeding of these transgenic medaka into the see-through strain (Wakamatsu *et al.*, 2001), in which the whole body is transparent and internal organs can be easily observed, will further improve the detection of GFP.

These biomonitoring transgenic lines can be bred together to form double transgenic line to detect a wider range of pollutants. If different promoters are linked to different fluorescent protein, it is possible to detect different classes of pollutants by observing the types of fluorescence expressed in a single double transgenic line.

In addition as an indicator for PAH and dioxin aquatic pollution, Tg(*cyp1a:gfp*) could be used for drug screening. As drug-drug interaction is important in drug design, it is essential to study drug metabolism by cyptochrome P450 enzymes as well as the induction/inhibition of other cyptochrome P450 enzymes (Pelkonen *et al.*, 2008). CYP1a2 is one of the listed CYP enzymes in the draft guidance by FDA for drug interaction studies (Huang *et al.*, 2007). Since *cyp1a* is the only orthologue in medaka compared to human *CYP1a1* and *CYP1a2*, Tg(*cyp1a:gfp*) could be used to indicate possible drug induction of human CYP1a2. Assay using Tg(*cyp1a:gfp*) fish embryo could be high throughput and rapid, not forgetting that it is relatively cheap and needs low maintanence. As Tg(*cyp1a:gfp*) represent physiological model, assay using Tg(*cyp1a:gfp*) fish larvae could either replace the role of cell culture studies or as additional step before progressing to mammalian in vivo studies.

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