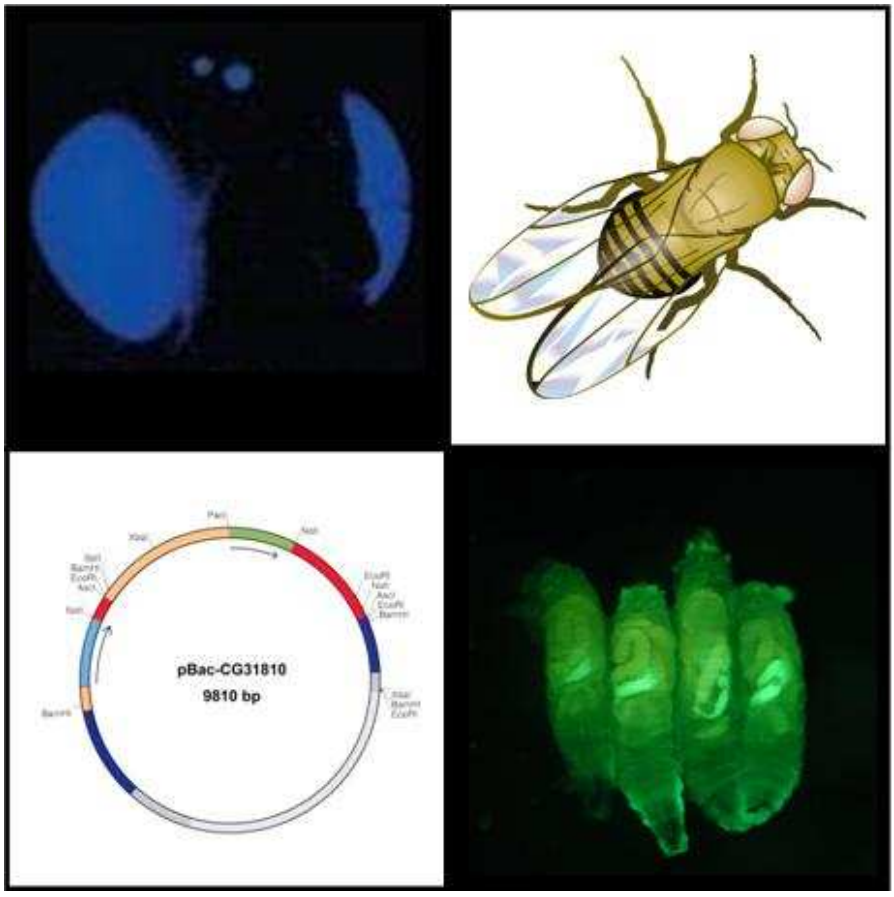
DRUGS FROM FLIES

Development of an *in vivo* *Drosophila*-based screening assay for antioxidant compounds



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Dianne van den Heuvel
Supervisors: Dr. Maarten Jongsma (Plant Research International)
Dr. Joop van Loon (Laboratory of Entomology)

PLANT RESEARCH INTERNATIONAL
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Development of an *in vivo* *Drosophila*-based screening assay for antioxidant compounds

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Front page pictures:
Top left: ***Expression ECFP in the eyes of*** **Drosophila** ***(Horn & Wimmer, 2000)***
Top right: **Drosophila** ***w1118 (white eyes)***
Bottom left: ***pBac-CG31810-EGFP vector***
Bottom right: ***Autofluorescence in*** **Drosophila** ***larvae (photo: D.M.A. van den Heuvel)***

Preface and acknowledgement

This is the report of my first thesis project, which is part of my MSc Cell Biology at the Wageningen University. The research project was carried out at Plant Research International (PRI) in Wageningen under supervision of the Chair Group Entomology.
The research project, the development of an *in vivo Drosophila*-based screening assay for antioxidant compounds, is a new research project at PRI. As a student, I found it very challenging to start this new project. It made me feel like a real researcher having my very own project! It was very exciting to develop some research methods myself, by performing accurate literature research and contacting experienced researchers all over the world.
This was a very good experience. I learned a lot from it!
Another reason why I liked the project very much is that it combined different research disciplines: molecular biology, the construction of DNA vectors, and cell biology/ genetics, by performing *Drosophila* germline transformation. I liked this all very much! I got really fascinated by the DNA itself and the process of germline transformation.

I would like to thank my supervisor Dr. Joop van Loon and Prof. Marcel Dicke of the Laboratory of Entomology for their assistance.
I'm very grateful to my PRI supervisor Dr. Maarten Jongsma (Bioscience) for his helpful instructions, enthusiastic approach, the trust he gave me to develop my own research methods and the possibility to extend my project for 3 months. In addition, a lot of thanks to Ralph Litjens, who works as a technical assistant in the Bioscience group, for his true interest and above all for being a very good teacher in showing me how to work in the lab as a real molecular biologist! Thanks Ralph!
I would like to thank all other researchers of the Bioscience group and the Biointeractions group of Gerrie Wiegers for their help and contribution to the pleasant working atmosphere.
In particular thanks to all students at PRI for their friendship, joking and for that reason memorable lunch breaks.
Special thanks to Bram Cornelissen, whom I ran into in the Biointeractions lab, for the nice talks and support during my stay at PRI and for being my lovely boyfriend from then on.

Dianne van den Heuvel, September 2005.

Abstract

This report describes the pilot research performed on the development of an *in vivo Drosophila*-based screening assay for antioxidant compounds. Living *Drosophila* larvae, genetically transformed with a GFP-reporter construct, are the subject material in this assay, which has the objective to discover antioxidant compounds in a very cost-effective way.
From the results of a microarray study on the influence of the antioxidant compound curcumin on *Drosophila* gene expression, four genes were selected that were shown to have a highly upregulated expression upon curcumin induction: the Steroid Dehydrogenase (SDR) genes CG31809 and CG31810, the Glutathione-S-Transferase (GST) gene CG6776 and the Multidrug Transporter gene (SDR) Mdr50.
GFP-reporter constructs were made by ligating the promoter and terminator parts of the selected genes to EGFP. The GFP-reporter constructs were cloned in the *piggyBac* transformation vector which has an ECFP marker construct incorporated. Germline transformation was performed by injecting young *Drosophila* embryos with the *piggyBac* construct vector and the *piggyBac* helper vector encoding the piggyBac transposase protein. Fluorescence microscopy was used to screen for genetically transformed *Drosophila*.
In none of the screened offspring of injected larvae a significantly higher level of ECFP and EGFP fluorescence could be detected compared to the fluorescence observed in wildtype larvae. It is hypothesized that there were genetically transformed *Drosophila* present among the screened larvae expressing the fluorescent proteins, but that they escaped detection due to the high level of autofluorescence and the filtersets used. The autofluorescence was mainly emitted from ingested food and could not be properly controlled. The filtersets were not suitable for an adequate distinction between ECFP and EGFP fluorescence in this experimental set-up.
The use of the fluorescent protein combination of DsRed in the construct vector and EGFP in the marker vector and the application of two bandpass filters is proposed as a solution to solve both problems.

Abbreviations

CDS	Coding Sequence
CIP	Calf Intestine Phosphatase
Dros.	Drosophila
DsRed	*Discosoma* red fluorescent protein
ECFP	Enhanced cyan fluorescent protein
EGFP	Enhanced green fluorescent protein
EYFP	Enhanced yellow fluorescent protein
E. coli	*Escherichia coli*
GMO	Genetically Modified Organism
LB-medium	Luria-Bertani medium
LUMC	Leiden University Medical Centre
Mp	Miniprep
MQ	MilliQ
PCR	Polymerase Chain Reaction
O.D.	Optical Density
O/N	Overnight
pBac	piggyBac
PRI	Plant Research International
Rpm	Rounds per minute
MCS	MultiCloning Site
WUR	Wageningen University and Research Centre

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CH1 Introduction

1.1 General introduction

In current medicine plants are a very important source of drugs by producing a lot of different natural compounds with medicinal properties. Nevertheless, it is generally thought that plants contain many more compounds with medicinal properties that still await discovery. This report describes research to develop an innovative method to screen plant compounds for medicinal properties: an *in vivo Drosophila*-based screening assay for antioxidant compounds.

The fruit fly (*Drosophila melanogaster)* is chosen as model organism in this assay. An important advantage of *Drosophila* is that it is an extensively studied animal, for which the complete genome was released in 2000 (Adams et al., 2000). Analysis of the sequence demonstrated that more than 60% of the genes that are implicated in a variety of human diseases have orthologues in *Drosophila* (Bernards & Hariharan, 2001). This finding and additional research showed that elementary disease processes are conserved between humans and flies, which have made *Drosophila* an important research subject in the molecular biology of medicine. An additional advantage of *Drosophila* is that it is a small animal, which is easy and quick to breed under laboratory conditions.

A well known food ingredient is curcumin, a polyphenol found in the root of the plant yellow root (*Curcuma longa*). Curcumin is the compound that makes Indian curries yellow, but it is also known to have many properties of medical importance (Appendix I) of which most important are its antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic, antiviral and antiinfectious activities in organisms (Joe B., 2004). Curcumin is known to up-regulate genes expressing antioxidants, which can explain most of its medicinal properties. Antioxidants are chemicals that scavenge free radicals, which are oxidative products that can react with biological compounds leading to tissue damage. Free radicals arise during the physiological process of respiration by which inhaled and tissue incorporated oxygen oxidizes biomolecules.

The development of the *in vivo Drosophila*-based screening assay for antioxidant compounds is based on genes known to be induced by curcumin. During previous research at PRI, the response of *Drosophila* gene expression to curcumin was analyzed and compared to the human response. In this research curcumin was fed to flies and with the help of DNA microarrays the global gene expression was determined at discrete time points. The enhanced expression of particular genes that explain the antioxidant action of curcumin in mammalian cells was also found in *Drosophila* (M. Jongsma 2004, pers com). These genes belong to two different gene families: Glutathione-S-Transferases (phase 2 genes) and Multidrug Transporter genes (phase 3 genes) (M. Jongsma 2004, pers com). However, members of the Steroid Dehydrogenases were upregulated the most strongly (100-fold) in *Drosophila*, whereas this was not previously described for mammals, possibly because no one studied the gene before. Presently, only the process of up-regulation of phase 2 genes is fully understood at the molecular level. Curcumin was shown to be able to disrupt complexes of the protein Keap1 and transcription factor Nrf2 (Sharoni, 2002). Nrf-2 is known to interact with the anti-oxidant responsive genetic elements resulting in the expression of most phase 2 genes.

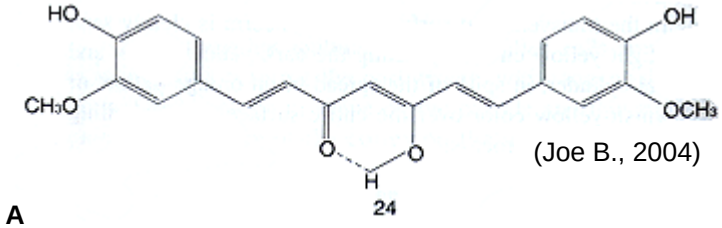
The results of the microarray studies were used in this project to develop an *in vivo Drosophila*-based screening assay for antioxidant compounds. GFP transgenic reporter systems are made utilizing the different genes known to be strongly regulated by curcumin, by fusing the promoter DNA sequences to the sequence of Green Fluorescent Protein (GFP). These constructs are incorporated in the *piggyBac* insect transformation vector. Germline transformation is performed by microinjection of the construct in very young *Drosophila* embryos. The larvae of the different resulting transgenic lines are fed with

curcumin and expression of GFP is made visible by UV microscopy. It is hypothesized that the level of expression of the antioxidant proteins resembles the expression level of EGFP, which is reflected in the fluorescence intensity. In such an assay system determination of the fluorescence intensity is thought to give a reliable indication of the medicinal potential of a certain plant compound tested.

1.2 Curcumin

1.2.1 Curcumin and its antioxidant properties

Curcumin ($C_{21}H_{20}O_6$) is found in the plant yellow root (*Curcuma longa* L., fam. *Zingiberaceae*). It is present in the natural yellow pigment in the tuberous root of the plant that is referred to as turmeric (Chattopadyay et al., 2004). In Asian countries turmeric has been used for centuries as a spice, food preservative and colouring agent. The yellow pigmented fraction of *Curcuma longa* contains a group of polyphenol compounds called curcuminoids that includes curcumin. Curcuminoids represent 3-5% of turmeric weight (?). The three main curcuminoids isolated are: curcumin, demethoxycurcumin and bisdemethoxycurcumin (Joe, 2004).



(Joe B., 2004)

A

B

Figure 1: A) Chemical structure of curcumin B) tuberous root of yellow root (Curcuma longa)

Curcuminoids are known to exhibit a variety of beneficial effects on health, partly by positively influencing events that help in preventing certain diseases. Curcumin is the most active ingredient responsible for the biological activity of turmeric and has been shown to have a wide spectrum of biological actions of which most important are its antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic, antiviral and antiinfectious activities (Appendix I). Safety evaluation studies indicated that both turmeric and its main compound curcumin are well tolerated at a high dose without any toxic effects (Chattopadyay et al., 2004). In view of the recently discovered applications, the properties of curcuminoids can be best summarized as being protective. Curcuminoids can exert protection either directly, by shielding biomolecules or indirectly by stimulating the natural detoxification and defence mechanisms of the body (Joe, 2004).

The antioxidant properties of curcumin are believed to explain many of its wide ranging pharmacological activities. It is an effective antioxidant that scavenges superoxide radicals, hydrogen peroxide and inhibits the inducible nitric oxide synthase activity in macrophages. As an antioxidant molecule curcumin not only exhibits antioxidative and free radical scavenging properties, but has also been shown to enhance the activities of other antioxidants, as for example superoxide dismutase, catalase and glutathione peroxidase (Joe, 2004).

Free radical mediated damage to biological systems is recognized as the initiating agent for many diseases. Throughout the years diverse research projects stressed the important antioxidant properties of curcumin. For example, curcumin was shown to protect the molecule haemoglobin from oxidation (Unnikrishnan & Rao, 1995). Curcumin was also found to protect renal cells and neural glial cells from oxidative stress (Cohly et al., 1996). In an experiment in which rats got an oral administration of 30 mg/kg body weight of curcumin for 10 days this was shown to result in a significant reduction of iron-induced hepatic damage by lowering of the rate of lipid peroxidation (Reddy & Lokesh, 1996).
The antioxidant property of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and an enol form of ß-diketone (Chattopadyay I. et al., 2004).
The non-enzymatic antioxidant activity of curcumin is thought to be mediated by a process of two steps (Figure 2). In Figure 2, S is the substance oxidized, AH is the phenolic antioxidant, $A^{\cdot}$ is the antioxidant radical and $X^{\cdot}$ is another radical species or the same species. In the first reaction step the oxidized substance S is reduced and the phenolic antioxidant becomes a weak free radical itself. In the second step this weak free radical dimerizes with another free radical $X^{\cdot}$ in order to form a non-radical product (Chattopadyay et al., 2004).

$$S\text{-}OO^{\circ} + AH \rightarrow SOOH + A^{\circ},$$
$$A^{\bullet} + X^{\bullet} \rightarrow \text{Nonradical materials}$$

Figure 2: Reaction scheme of the antioxidant action of curcumin (AH) on oxygen radicals (S-OO and X) (Chattopadyay I. et al., 2004)

The large number of benificial health properties associated with curcumin make it a very special and interesting molecule, which is thought to be of great promise for medical applications. In contrast, there are some researches that indicate that curcumin can also turn into a pro-oxidant molecule under certain circumstances and damage the DNA (Ahsan et al., 1999). Much research has to be done in order to elucidate all the different actions of this molecule at the molecular level.

1.2.2 Curcumin induced genes

1.2.2.1 *Drosophila* gene families induced by curcumin

In previous research at PRI the response of *Drosophila* gene expression to curcumin was analyzed by means of microarray studies. About a dozen genes belonging to three different gene families showed a strong to very strong (factor 5 – 70) elevated expression level after exposure of the larvae to a diet containing curcumin (Appendix X). These are the gene families of the steroid dehydrogenases, glutathione-S-transferases (phase 2 genes) and multidrug transporter genes (phase 3 genes) (M. Jongsma 2004, pers com).

1.2.2.2 Steroid Dehydrogenases

For the upregulated *Drosophila* steroid dehydrogenase genes high identity scores were found with the 17β -hydroxysteroid dehydrogenases from humans and mice. At present 11 distinct forms of 17β-HSDs have been described in mammals and except for 17β-HSD5 they all belong to the family of Short Chain Dehydrogenases (SDRs).
These 17β-HSDs were shown to differ in their substrate specificities, to have different tissue, developmental and subcellular distribution patterns and in their preferred reaction direction in *vivo* (Illiopoulos et al., 2003). 17β-HSDs catalyse the oxidation/reduction of 17β-OH(-hydroxy)/17-oxo groups of steroids, which makes them essential in the mammalian hormone physiology (Shafqat et al., 2003). The mammalian 17β-HSDs form a large group of enzymes, which are involved in sex steroid metabolism and control of the hormone levels of for example estrogens and androgens (Illiopoulos et al., 2003).

1.2.2.1.2 Glutathione-S-Transferases (phase 2 genes)

Glutathione-S-Transferases (GST) are a family of enzymes that catalyze the addition of a tripeptide glutathione to endogenous and xenobiotic substrates with electrophilic functional groups. The glutathione adducts produced have an increased solubility in water and are subsequently enzymatically degraded and excreted (Ji et al., 1994). Because of this function GSTs play an important role in the detoxification and metabolism of many xenobiotic and endobiotic compounds.
Glutathione S-Transferases belong to the group of phase 2 enzymes, which also includes UDP-glucoronosyl transferases (UGT) and NAD(H):Quinone oxidoreductases (NQO). All these enzymes have the ability to detoxify compounds by conjugating them to a particular endogenous molecule.
The induction of phase 2 genes, encoding proteins that protect against damage of electrophiles and reactive oxygen intermediates, is thought to be a major strategy for reducing the risk of cancer and chronic degenerative diseases (Wakabayashi et al., 2004)

1.2.2.1.3 Multidrug Transporters (phase 3 genes)

Multidrug transporters (phase 3 gene) function as a defensive mechanism to protect cells for xenobiotic substances. Xenobiotics are generally lipophilic, which makes it possible for them to enter cells by passive diffusion through the cell membranes (Van Veen, 1997). Multidrug transporters are located in the cell membranes, where they transport xenobiotic molecules out of the cell. The family of multidrug transporters genes is recently characterized as phase 3 genes.

1.2.2.2 Co-expression of curcumin induced genes

It has been shown that groups of co-expressed genes identified with microarray experiments often share similar types of *cis*-regulatory elements (Nees, 2001). In eukaryotes, modulation of gene expression is achieved through the complex interaction of regulatory proteins with specific DNA regions called the *cis*-acting regulatory elements. These c*is*-acting regulatory elements determine the level and timing of expression. Co-regulation is often mediated by a defined set of regulatory proteins interacting with a particular set of *cis*-acting regulatory elements.
One of the major pathways by which phytochemicals increase the expression of antioxidant and detoxification enzymes involves the *cis*-acting antioxidant responsive elements (ARE) found in the

promoters of these inducible genes (phase 2 genes) (Hayes, 2001). This gene induction is dependent on the Nuclear Factor-Erythroid 2p45-related factors, Nrf1 and Nrf2 (Sharoni, 2002).
Under basal conditions, these basic region leucine zipper transcription factors are located in the cytoplasm of the cell bound to Keap1. Upon challenge with inducing agents they are released from Keap1 and translocate to the nucleus. Within the nucleus, Nrf1 and Nrf2 are recruited to the ARE as heterodimers with either of the small Maf proteins, FosB, c-Jun or JunD, activating transcription factor 2 (ATF2) or ATF4. The consensus sequence of the ARE in the rat *GSTA2* promoter was initially defined by mutational analysis as 5`-TGACNNNGC-3` (Rushmore, 1991). Recent work came up with an improved version of the ARE, from the mouse *Gsta1* promoter: 5`-A/GTGAC/TNNNGCA/G-3` (Wasserman, 1997). The identification of ARE-enhancers in targeting genes is an initial step in the explanation of the molecular mechanism of the chemoprotective response by a certain phytochemical.

1.3 The *in vivo Drosophila*-based screening assay for antioxidant compounds

1.3.1 The principle of the *in vivo Drosophila*-based screening assay

Living *Drosophila* larvae, genetically transformed with a GFP-transgenic reporter construct, constitute the biological test material for this assay, which has the objective to discover antioxidant compounds. The GFP-transgenic reporter constructs consist of the promoter sequence of one of the *Drosophila* genes known to play an important role in the induction of the antioxidant reaction, fused to the sequence of Green Fluorescent Protein (GFP). The assay is used to verify whether a certain plant compound (P) has medicinal properties through stimulation of those particular genes (Figure 3). In the experiment which has to be performed, plant compound P is fed to X-GFP *Drosophila* larvae and EGFP expression is measured at certain time intervals. In this assay system the EGFP fluorescence intensity correlates with the expression level of the antioxidant protein and is a reliable indication of the medicinal potential of the plant compound tested.

An *in vivo Drosophila*-based screening assay for antioxidant compounds

Assay: Living *Drosophila* larvae, which are genetically transformed with a GFP-transgenic reporter construct

Construct: promoter gene **X** – GFP
The protein encoded by gene **X** has a positive health effect, by inducing an antioxidant action.

Question:
Does plant compound **P** induce an antioxidant action by stimulating the expression of gene **X**?

Experiment:
X-GFP larvae are fed with compound P and GFP expression is measured at different time intervals.

Result:
Strong expression of EGFP: compound **P** has a significant antioxidant effect.

Low expression of EGFP: compound **P** has a very low (possibly not significant) antioxidant effect.

No expression of EGFP: compound **P** does not have an antioxidant effect.

Figure 3: Principle of an in vivo* Drosophila-*based screening assay for antioxidant compounds

1.3.2 *Drosophila* as a model organism

Drosophila melanogaster is one of the most intensively studied organisms in science. Its complete genome was released in 2000 and direct homology was found between *Drosophila* genes and genes that affect human disease (Adams et al., 2000). Of all the genes known to affect human disease, 61% (177 out of the 289 studied) have *Drosophila* orthologues and in addition around half of all *Drosophila* protein sequences are similar to those of mammals. For this reason *Drosophila* has become a model system for the investigation of many developmental and cellular processes common to higher eukaryotes, including humans (Adams, 2000). It provides the foundation for rapid analysis of some of the basic processes involved in human disease.

As a test animal *Drosophila* is small in size and is easy and quick to breed under laboratory conditions. There are four different stages in the life of the fruit fly: egg, larva, pupa and adult (Figure 4). At 21℃ a fresh culture of *D. melanogaster* will produce new adults in two weeks. Under these culture conditions the egg stage will take one day, the larval stages will take seven days and the pupal stage will take six days. The day after the egg is laid the larva hatches. The larva molts twice. During the periods of growth before and after molting, the larva is called an instar. Metamorphosis occurs in the puparium and the pupa starts to darken and the folded wings and pigment in the eyes becomes visible through the puparium just prior to the eclosion of the adult fly. After eclosion the fly is light in colour at first, the wings are unexpanded and the abdomen is long. In a few hours the wings expand, the abdomen becomes more rotund and the colour gradually darkens (Flagg, 1988). Food is easy to prepare with basic ingredients, sugar, corn meal and yeast and instant food can be bought from different companies. For genetic crosses it is easy to discern between male and female flies with the naked eye. Males are generally smaller and have a darker and more rounded abdomen and in addition males have tarsal sex combs on their first pair of legs. Male flies have genital arches which are slightly red colored.

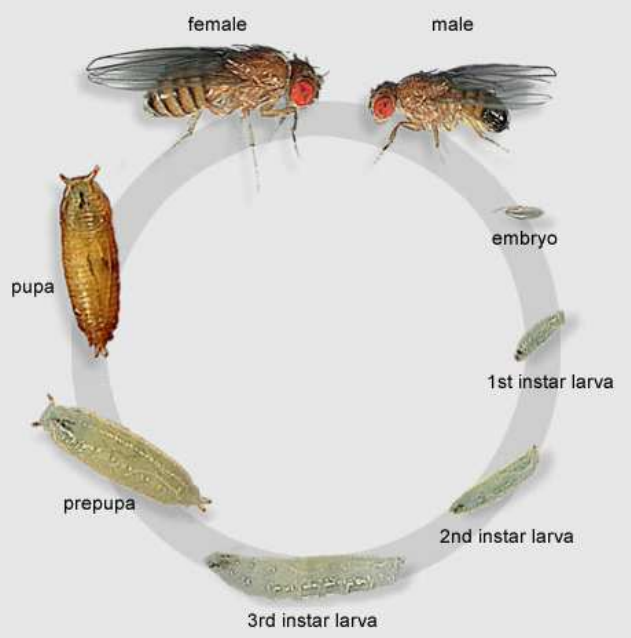


Figure 4: Life cycle of Drosophila melanogaster.

1.3.3 piggyBac vector

Germline transformation of insects was first demonstrated in *Drosophila melanogaster* by the use of the transposable element P. P-element transformation has been a routine technique for *Drosophila* research for more than 20 years (Rubin and Spradling, 1982). For the reason that the P transposon is inactive in non-drosophilids, transformation systems with vectors containing other transposons were developed, like the *hermes*, hobo, *mariner*, *minos* and *piggyBac* transposons (Toshiki et al., 2000).
PiggyBac is the most widely used transposable element vector system for the germline transformation of insects (Handler A.M., 2002). This vector system consists of a construct vector and a helper vector encoding the *piggyBac* transposase (Figure 5). *piggyBac*-derived vectors have been shown to be capable of mediating germline transformation in a wide variety of insect species, as for example in the medfly *Ceratitis capitata*, the mosquito *Aedes aegypti*, the silk moth *Bombyx mori*, the ladybird *Harmonia axyridis* and the flour beetle *Tribolium castaneum*, indicating that it can be used as a universal insect transformation vector (Toshiki et al., 2000).
The *piggyBac* transposable element was originally isolated from a Lepidopteran cell line of *Trichoplusia ni* as a gene-disrupting insertion within spontaneously arising baculovirus plaque morphology mutants (Cary *et al*., 1989). The *piggyBac* transposable element inserts specifically at TTAA *piggyBac*. The *piggyBac* transposase encoded by the *piggyBac* helpervector (Figure 5) operates using a precise cut-and-paste mechanism, targeting the TTAA sites, which are duplicated upon insertion and are crucial to proper excision reforming a single target site (Cary *et al*., 1989). The *piggyBac* vector has a single transcription factor-activated artificial promoter and insertion in the genome is essentially random with respect to the fact that gene sequences are targeted as frequently as flanking DNA (Cary et al., 1989). DNA cloned between the terminal repeats (piggyBacL and piggyBacR in Figure 5) of the *piggyBac* vector is introduced into the genome by the catalyzing action of the *piggyBac* transposase. The *piggyBac* transposase is encoded by a separate helper plasmid, which has no sequences for integration into the genome.

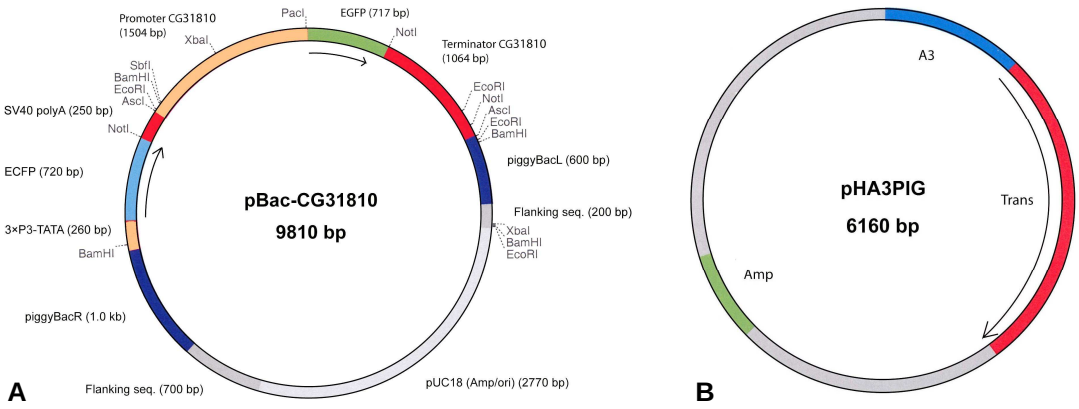


Figure 5: A) piggyBac construct vector (piggyBacR & piggyBacL: terminal repeat sequences, 3×P3-TATA: photoreceptor specific promoter, ECFP: enhanced cyan fluorescent protein, SV40 poly A: SV40 polyadenylation sequence, Promoter CG31810: promoter sequence of Drosophila gene CG31810, EGFP: enhanced green fluorescent protein, Terminator CG31810: terminator sequence of Drosophila gene CG31810. B) piggyBac helper vector (Amp: ampicilin gene, Trans: transposase, A3: viral late A3 promoter)

1.3.4 Fluorescent markers

In this research two fluorescent proteins are used as marker molecules: enhanced cyan fluorescent protein (ECFP) and enhanced green fluorescent protein (EGFP). EGFP is a red shifted GFP-variant with an excitation peak in the blue light zone at 488 nm, which is more harmless than the UV-light used to excite GFP (Horn et al., 2002). Another advantage of EGFP above GFP is that EGFP has a 35 times higher fluorescence intensity (509 nm) compared to GFP. ECFP is a more bluish mutant of CFP has excitation and emission maxima of 434 and 477 nm, respectively.
EGFP is the fluorescent marker used in the GFP-reporter system and ECFP is the independent transformation marker. EGFP is used in the GFP-reporter system, because it is a more sensitive marker than ECFP and exhibits much less autofluorescence when excited with short wavelength blue light than ECFP (Horn et al., 2002).
ECFP is used as an independent transformation marker, for which expression is regulated by an artificial promoter containing three Pax-6-homo-dimer binding sites in front of a TATA box. Pax-6/Eyeless is a transcription factor and the master regulator of eye development in *Drosophila* and also in vertebrates (Horn et al., 2000). The 3×P3-ECFP gene construct drives strong photoreceptor-specific expression of ECFP in the eye-tissues, but also in the central and peripheral nervous anal paths of larvae, pupa and adult flies (*Figure 6*).
The fluorescent markers EGFP and ECFP are much more sensitive transformation markers than the *Drosophila* 'mini-white' transformation marker, which is based on the reversion of a mutant white eye colour to red. Another disadvantage of the 'mini-white' transformation marker is that it is more receptive to slight position effects.
Both fluorescent proteins are quite small (27 kDa) and can freely enter the nucleus and fill the cytoplasm of the cell. Toxicity effects are never observed for these fluorescent proteins, also not for 3×P3-ECFP even when high expression causes intense fluorescence in the eyes and central nervous system (Horn et al., 2002).

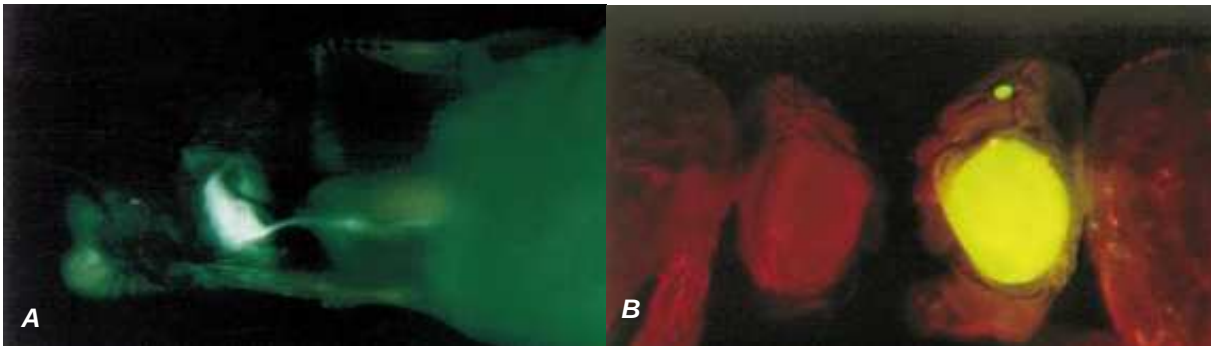


Figure 6: EGFP fluorescence can be observed in the larval eyes and in the optic nerve in a 3×P3-EGFP transformed* Drosophila *larvae (A) and in the complete compound eye and the ocelli of an a 3×P3-EGFP transformed* Drosophila w1118 *fly (B) (Berghammer et al., 1999).

1.3.6 Germline transformation by microinjection

Microinjection is the most efficient insect transformation technique to date (Horn et al., 2002) and will be used in this research project for the germline transformation of *Drosophila*. This method takes advantage of the way in which *Drosophila* makes germ cells. The early embryo divides as a plasmodium, resulting in the presence of many nuclei in a common cytoplasm. The nuclei migrate to the cortex of the egg, where they are enclosed by membranes to form cells. The germ line determinants of these cells are localized at the posterior cortical cytoplasm of the egg. For this reason DNA injected into the posterior cortical cytoplasm of the egg can be incorporated into the newly forming germ cells. Rubin and Spradling developed the microinjection method for germline transformation using transposable elements (Rubin and Spradling, 1982).
In order to get germline transformation, the following events must take place. The injected plasmids, construct vector and helper vector, should be taken up by the germline cells. The helper plasmid should enter the nucleus in order to get transcribed and the resulting mRNA has to become translated into a transposase protein in the cytoplasm. Subsequently, also the transposase has to enter the nucleus where it catalyzes the insertion of one or more *piggyBac* construct elements into the chromosomes.
For an efficient transformation it is very important that the *piggyBac* construct and helper vector are purified from *E. coli* by means of Endofree maxi plasmid prepping. An Endofree maxi plasmid prep removes the endotoxines, also known as lipopolysaccharides or LPS, which are cell membrane components of Gram-negative bacteria. The lipid portion of the outer layer of the outer membrane is completely composed of these endotoxin molecules. Endotoxins are known to strongly influence the transfection of DNA into cells and lead to sharply reduced transfection efficiencies (Weber et al., 1995).

1.3.7 Control experiments

The expression of ECFP of which the gene sequence is present in all *piggyBac* construct vectors is the positive control to verify whether the germline transformation technique is successful. ECFP is thought to be independently expressed from EGFP. In case of successful germline transformation ECFP fluorescence should be visible and be independent of the expression of the EGFP construct.
In order to verify which proportion of the observed fluorescence results from the expression of the 3×P3-ECFP marker gene and which part from the EGFP-reporter construct a negative control experiment is the transformation of *Drosophila* embryos with the 'empty' pBac[3×P3-ECFPamf] vector, without EGFP-reporter construct.
To verify the influence of DNA injection itself on the survival rate of *Drosophila* larvae and flies a negative control experiment is the execution of the whole microinjection protocol without DNA injection itself.

1.4 Objective

The goal of this research project is to develop an *in vivo Drosophila*-based screening assay for antioxidant compounds. Such an assay is thought to be highly cost-effective compared to current gene expression screening methods that utilize cell lines or rodents to evaluate compounds. It is expected that the amount of laboratory animals can be reduced by using a *Drosophila*-based screening assay while getting the same reliable results. In addition, only very small amounts of the test compounds are needed. In this way, a systematic approach to the screening of compounds is possible in a very cost-effective and rapid way. This is thought to be the starting phase in the discovery of new medicines, which is followed by validation of the observed effects in an animal system. The primary goal of this research is to verify whether it is possible to develop such an assay. The main research question is: Is an *in vivo Drosophila*-based screening assay suitable for the detection of antioxidant compounds? The hypothesis in this research is the confirmation of the research question.

CH2 Materials and methods

2.1 The GFP-reporter piggyBac construct vectors

2.1.1 Standard molecular techniques

Restriction enzymes and other enzymes used for DNA manipulations were purchased from Invitrogen or New England Biolabs and used according to the supplier's instructions.The QIAprep Spin Miniprep Purification Kit (Appendix XV) was used for the isolation of plasmid DNA, the JETQUICK Gel Extraction Spin Kit (Appendix XIV) was used for the isolation of DNA fragments from agarose gels and PCR products were purified using the JETQUICK PCR purification Spin Kit (*Appendix N*), following the supplier's instructions
Genomic DNA isolation of *Drosophila* was performed by the 'Rapid DNA extraction from ferns for PCR-based analysis' as described by Dempster et al., 1999 (Appendix VIII). Competent cells are prepared according to the protocol of Inouye et al., 1990 (Appendix IX).

2.1.2 *Drosophila* gene selection

For the construction of the GFP-reporter constructs four genes belonging to three different gene families were chosen out of the *Drosophila* genes in the microarray study which were shown to have a highly upregulated expression after curcumin induction *(*Appendix II*).* These are the steroid dehydrogenase (SDR) genes CG31809 and CG31810, the glutathione-S-transferase (GST) gene CG6776 and the multidrug transporter (MDR) Mdr50.
The DNA sequences of these genes are online available in the Flybase (h*ttp://flybase.bio.indiana.edu).* A sequence similarity investigation was performed with the Multialign program (http://www.toulouse.inra.fr/multalin.html). The presence of potential ARE enhancer sequences in the promoter were investigated with the Editseq program.

2.1.3 *E.coli* culture

The *E. coli* XL1-Blue MRF`strain (Stratagene) was used for the propagation of all plasmids. The *E. coli* XL1-Blue strain was routinely grown in Luria-Bertani (LB) medium at 37˚C with ampicillin as an antibiotic.

2.1.4 PCR and vector construction

Primers were designed in Clone Manager in order to amplify the EGFP-gene with PCR and to isolate the promoter and terminator sequences of the selected *Drosophila* genes by nested PCR. The sizes of the different promoter and terminator sequences *(*Appendix IV*)* were determined after examination of the position of neighbouring genes in the DNA sequence *(*Appendix V*)*. The EGFP forward primer was designed to incorporate a Kozak consensus sequence which flanks the AUG initiator codon and

modulates translation by eukaryotic ribosomes. All PCR reactions are performed with the PTC-200 PCR machine (Biozym).
The EGFP-gene was amplified out of the EGFPAVI2-3 plasmid with the DNA polymerase Pfu polymerase (Stratagene) and *NotI/PacI* cloned into the PSL-Gal4 shuttle vector (Appendix IV-A). The DNA polymerase SuperTaq (SphaeroQ) was used to isolate the different promoter and terminator sequences of the four selected genes out of the genomic *Drosophila* DNA. Because of its rather big size (4137bp), the promoter of gene CG31809 was isolated with the expand high fidelity PCR system (Roche), which is composed of a unique enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase. PCR fragments were separated on a 0.8% agarose gels and isolated with the JETQUICK Gel Extraction Spin Kit (Appendix XIV).
In order to constitute the four GFP-reporter constructs the different terminator DNA sequences were cut with *NotI* and ligated in the *NotI* site of the PSL/Gal4-vector (Appendix IV-A) in different reactions. The different promoter DNA sequences were cut with *SbfI* and *PacI* and *SbfI*/*PacI* ligated into the PSL/Gal4-vector already containing the gene specific terminator sequence. An exception is the CG31809 promoter which was only cut with *SbfI* and ligated in the *SbfI*-site of the PSL/Gal4-vector already containing the terminator of gene CG31809.
Finally, the different GFP-reporter construct *piggyBac* vectors were fabricated by *AscI* digestion of the GFP-reporter constructs in the PSL/Gal4 shuttle vectors and ligation into the *AscI*-site of the *piggyBac* vector pBac[3×P3-ECFPamf] (Appendix IV-B). An overview of the pBac vector construction is given in Appendix III.

2.1.5 Endonuclease reactions

After each cloning step in the construction process of the different *pBac* construct vectors the presence of the correct DNA fragment and if necessary its orientation were verified with appropriate endonuclease digestions resulting in restriction fragments of specific predicted sizes.
The correct composition of the final products, the GFP-reporter construct *pBac* vectors, was similarly verified with different endonuclease reactions. To determine the orientation of the *AscI/AscI* GFP-reporter constructs in the pBac vectors, the pBac vectors were cut with *EcoR*I. In addition, the pBac-G31810-EGFP vector was also cut with *XbaI*. These restriction reactions also give important information about the composition of the GFP-reporter constructs.
The pBac vectors were also cut with *BamHI*, which cuts the whole intact GFP-reporter construct in case of correct orientation out of the pBac-vectors. In theory, the GFP-reporter construct pBac vectors have 4 *BamHI* restriction sites. One is situated in the multicloning site (MCS) flanking the inserted GFP-reporter construct at its 3' end. The other restriction site is located in the construct itself (5' end) in a small piece of DNA adjacent to the gene promoter sequence which originates from the MCS of the PSL-vector. *BamHI* has no recognition sites in the remaining part of the GFP-reporter construct. *BamHI* restriction is an additional test to determine whether the GFP-reporter construct pBac vectors have the correct GFP-reporter construct sequence incorporated in the correct orientation.

2.1.5 DNA sequencing

DNA sequencing (Appendix VII) of EGFP was performed after the first cloning step of *NotI* ligation of EGFP into the PSL/GAL4 vector, with primers located at the 3' and 5' end of the EGFP sequence (Appendix VI). In the GFP-reporter construct pBac vector EGFP was sequenced for a second time, with gene specific primers at the promoter 5' end and terminator 3' end primers (Appendix VI). The EGFP primers could not be used because they would give interference problems with the homologous ECFP gene present in the pBac vector.
The 5' end of the construct specific promoter and the 3' end of the construct gene terminator were sequenced in the GFP-reporter construct PSL/Gal4 vector, with the EGFP primers.

2.2 *Drosophila* germline transformation by microinjection

2.2.1 Fly rearing

The *Drosophila melanogaster w 1118* (white eyed) strain was obtained from the research group of J.N. Noordermeer, LUMC Leiden. The *w1118* fly strain was raised at 24˚C, a 6h:8h light/dark schedule and fed on a standard corneal-brown sugar-agar-yeast (Appendix XX-AI). Larvae and flies developing from injected eggs were kept in a GMO culture cell.

2.2.2 Collection and preparation of embryos

Eggs were collected by letting adult flies (2-7 days old) lay eggs in 3-5 egg collection cages (approx. 100 flies per cage) for 15 min. The eggs are collected in an 'epp with a mesh' and flushed with Triton-X100 (0.01%). Dechorionation of the embyros is performed by holding the embryos 45 s in 50% bleach. The embryos were aligned by inspecting the anterior-posterior orientation, on a small strip of agar (5×50 mm) using a binocular with cold light (0.8×), and transferred to a coverslip with a thin layer of untoxic glue (3M Scotch double sided tape 415) by lightly pressing the coverslip onto the aligned embryos. The embryos were dehydrated in a box with silica gel for 10 min and subsequently covered with 10s Voltalef oil (fabricant??).

2.2.3 Injection solution preparation

The *piggyBac* construct and helper vector were purified by means of an Endofree maxi plasmid prep (Appendix XVII) and dissolved in injection buffer (pH 7.8; 10 mM $NaPO_4$ & 0.5 mM KCl) resulting in a final concentration of 600 ng/ µl for the pBac construct vector and 400 ng/ µl for the pBac helper vector (Appendix XIX).

2.2.4 Microinjection

Microinjections were performed using an Eppendorf microinjection system, with parameters: P_i 500-70 hPa, T_i 0.5s, P_c 30-70 hPa. The Femtotip needles were held by a very simple micromanipulator Narishige (MN-151). The embryos were injected by moving the microscope stage, injection itself was activated by a mouse click. Only embryos of very young stages were injected by penetrating the needle into the embryo in a quick manner for at maximum one-fourth of its length and injecting a drop of 1/10th of the embryo size (Appendix XXI). The injections were carried out at room temperature (approx. 22 ˚C). Five different injection solutions with the different construct vectors pBac-CFP-[C09], pBac-CFP-[C10], pBac-CFP-[C50], pBac-CFP-[C76] and the original pBac vector without GFP reporter construct pBac[3×P3-ECFPamf] were used.
The 'empty' pBac[3×P3-ECFPamf] vector, without GFP-reporter construct was injected to verify which proportion of the observed fluorescence resulted from the expression of the 3×P3-ECFP marker gene and which part from expression of the EGFP-reporter construct.
In order to verify the influence of DNA injection itself on the survival rate of *Drosophila* larvae the whole microinjection protocol is also executed without performing DNA injection.

2.2.5 Post-injection care

The coverslip with injected embryos is kept in a closed petridish with grape juice agar and a little spot of yeast on the lab bench. Hatched larvae were collected from the yeast after approx. 40h. Larvae originating from the injected embryos (G_0) were transferred to a fly vial with standard food (Appendix XX-AI). Developed G_0 flies were crossed by putting 2-4 G_0 flies of the same sex together with 4-6 *w1118* flies of the opposite sex in a fly vial with semi-defined medium to which curcumin (2 mg/g) is added (Appendix XX-C). The survival rate of both G_0 larvae and adult flies relative to the number of injected eggs was determined.

2.2.6 Fluorescence screening

All potentially transformed G_1 larvae were screened for ECFP and EGFP fluorescence and a certain number of G_1 adult flies was checked for ECFP fluorescence in the eyes. Fluorescence was investigated with a Leica MZ FLIII fluorescence stereomicroscope and filters: GFP1 (excitation filter 425/60 nm, barrier filter 480 nm), GFP2 (excitation filter: 480/40 nm, barrier filter 510 nm) and GFP 3 (excitation filter: 470/40 nm, barrier filter 525/50 nm). Live flies were anesthetized with CO_2 and kept on ice for observation.
For ECFP fluorescence the larval and adult eyes and the larval nerve system were checked with the GFP1-filter. Screening for EGFP fluorescence was performed by observing larvae through all 3 filters, preferably filter 2. Pictures were taken with a Nikon Coolpix 990 (E990V1.1) camera.

CH3 Results

3.1 Analysis of the gene promoters and terminators

3.1.1 Sequence homology

In the sequence homology search a high degree of sequence similarity is detected between the promoters of the steroid dehydrogenase genes CG31809 and CG31810. Gene CG31809 is positioned upstream of gene CG31810 and its promoter (4137 bp) is much longer than the promoter of gene CG31810 (1504 bp). Figure 7 shows the homologous parts between the two gene promoters.

Figure 7: Sequence homology promoters CG31809 and CG31810 (3'→5' orientation)

3.1.2 Investigation of the presence of ARE enhancers

In the promoter and terminator sequences of the selected genes the antioxidant responsive element (ARE) consensus sequence: 5'- A/G TGA C/T NNNGC A/G - 3' (Hayes & McMahon, 2001) could not be identified in its strict form. However, very homologous sequences could be detected differing in only 1 to 3 nucleotides from the consensus sequence (Appendix XXIII). In the promoter sequences of both steroid dehydrogenase genes some good candidates are found: 4 in gene CG31809 (1/1007) and 2 in gene CG31810 (1/707). In addition, also in the terminator sequences of these genes potential ARE sequences could be identified, 1 in gene CG31809 and 2 in gene CG31810. In the promoter of the Mdr50 gene 8 potential ARE sequences are found (1/246). In the promoter of the glutathione S-transferase gene no potential ARE sequences could be identified.

3.2 The pBac GFP-reporter vectors

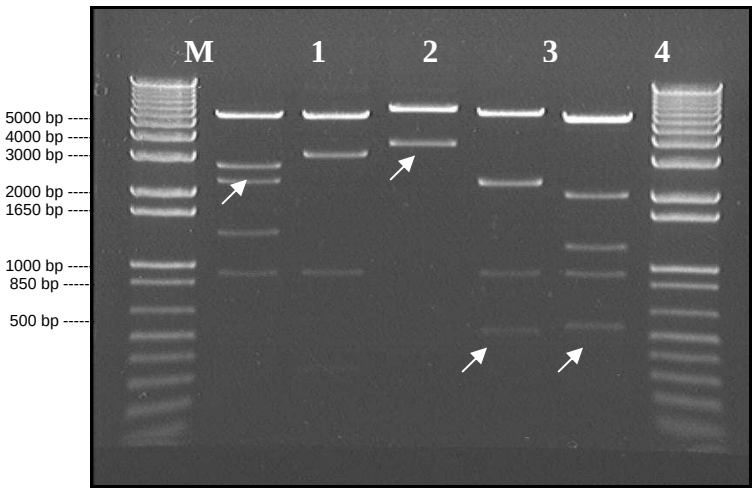
3.2.1 *EcoRI* and *XbaI* restriction of the pBac reporter-EGFP vectors

In Figure 8 the DNA fragments resulting from the *EcoRI* restriction of all GFP-reporter construct pBac vectors and *XbaI* restriction of the pBac-G31810-EGFP vector are visible after gel electrophoresis.
Table 1 shows the theoretical sizes of the restriction fragments of the different pBac vectors containing the GFP-reporter constructs in the correct (in bold) and incorrect orientation. The restriction fragments highlighted in grey are determinative for the distinction in correct and incorrect construct orientation, because a fragment of that particular size is only present in vectors with the GFP-reporter construct integrated in the correct orientation.

The sizes of the restriction fragments of the different pBac vectors on gel completely correspond to the expected sizes of the restriction fragments of the pBac vectors containing the GFP-reporter construct in the correct orientation, except for the *EcoRI* restriction of the pBac-CG31810-EGFP in which the expected 286 bp fragment is not visible. However, a second restriction reaction of pBac-CG31810-EGFP with *XbaI* results in two DNA fragments of which one is indicative for the pBac vector with correct construct orientation.

Table 1: Theoretical sizes of the DNA fragments after* EcoRI *and* XbaI *restriction of the GFP-reporter construct pBac vectors. Restriction fragments containing the GFP-reporter construct in the correct orientation are in bold. The indicative fragments are highlighted in grey.

CONSTRUCT	ENZYME	RESTRICTION FRAGMENTS
pBac-CG31809-EGFP	*EcoRI*	**2220 bp 925 bp 5566 bp 1342 bp 2646 bp**
pBac-CG31809-EGFP	*EcoRI*	10 bp 925 bp 6492 bp 2646 bp 1342 bp
pBac-CG31810-EGFP	*EcoRI*	**5549 bp 3050 bp (286 bp) 925 bp**
pBac-CG31810-EGFP	*EcoRI*	5825 bp 3050 bp 10 bp 925bp
pBac-CG31810-EGFP	*XbaI*	**3485 bp 6325 bp**
pBac-CG31810-EGFP	*XbaI*	1475 bp 8335 bp
pBac-Mdr50-EGFP	*EcoRI*	**527 bp 925 bp 5603 bp 1181 bp 1971bp**
pBac-Mdr50-EGFP	*EcoRI*	10 bp 925 bp 6120 bp 1181bp 197 bp
pBac-CG6776-EGFP	*EcoRI*	**500 bp 925 bp 5578 bp 2221 bp**
pBac-CG6776-EGFP	*EcoRI*	10 bp 925 bp 6068 bp 222 bp



Legenda Figure 8:

M Marker
1 pBac-CG31809-EGFP (*EcoRI*)
2 pBac-CG31810-EGFP (*EcoRI*)
3 pBac-CG31810-EGFP (*XbaI*)
4 pBac-CG6776-EGFP (*EcoRI*)
5 pBac-Mdr50-EGFP (*EcoRI*)
6 pBac[3×P3-EGFPamf] (*EcoRI*)
M Marker

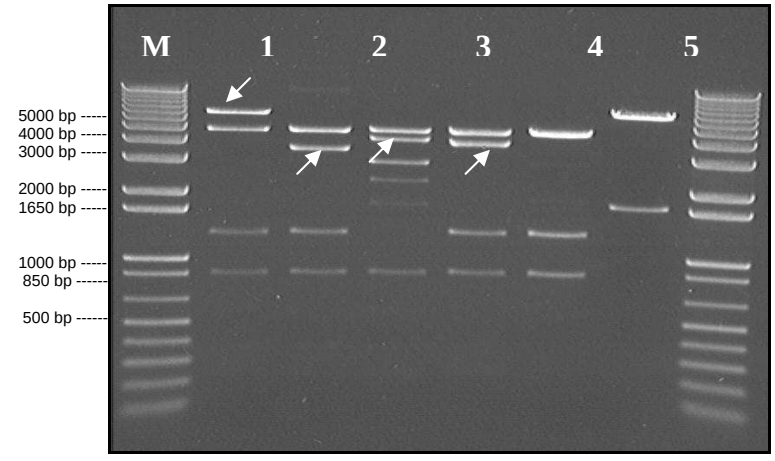
Figure 8: Result of* EcoRI *and* XbaI *restriction of the reporter-EGFP construct pBac vectors (1 µl of a 1:10 mp dilution, 1% agarose gel). White arrows indicate the indicative fragments for correct GFP-reporter construct orientation.

3.2.2 *BamHI* restriction of the GFP-reporter construct pBac vectors

Figure 9 shows the DNA fragments resulting from *BamHI* restriction of all GFP-reporter construct pBac vectors. *BamHI* restriction is a second test to determine whether the different pBac vectors have a correct composition. If the the pBac GFP reporter construct has the correct orientation digestion with *BamHI* cuts the whole fragment out of the pBac vector. Wrong orientations result in smaller fragments. Thus, it can be determined whether the different pBac constructs have incorporated the correct EGFP-reporter constructs in the correct orientation.
In Table 2 the DNA fragment sizes resulting from *BamHI* restriction of the different pBac vectors are listed, predicted from the theoretical DNA sequence. The fragment sizes in bold are the *BamHI/BamHI* cut GFP-reporter constructs.
Among the DNA fragments resulting from *BamHI* restriction of the different pBac vectors, one fragment corresponds to the expected size of the *BamHI/BamHI* cut GFP-reporter construct (Figure 9 white arrows). The sizes of the other resulting restriction fragments all correspond to the expected sizes of the remaining fragments, except for the restriction fragments resulting from the Bac-CG6776-EGFP vector digestion. In this restriction reaction result (Figure 9 lane 3) the expected fragment of 1300bp is missing and additional bands show up of resp. 1700bp, 2200 bp and 3000bp. The *BamHI* restriction result (Figure 9 lane 6) of the pBac helpervector pHA3PIG does not give any useful information, because there was no information available about the position of the different restriction sites in this vector.

Table 2: Theoretical sizes of the DNA fragments resulting from* BamHI *restriction of the GFP-reporter construct pBac vectors. In bold the size of the* BamHI-BamHI *promoter-EGFP construct.

pBac-CG31809-EGFP	*BamHI*	**6208 bp** 4350 bp 1300 bp 850 bp
pBac-CG31810-EGFP	*BamHI*	**3310 bp** 4350 bp 1300 bp 850 bp
pBac-Mdr50-EGFP	*BamHI*	**3707 bp** 4350 bp 1300 bp 850 bp
pBac-CG6776-EGFP	*BamHI*	**2724 bp** 4350 bp 1300 bp 850 bp



Legenda Figure 8:

M Marker
1 pBac-CG31809-EGFP
2 pBac-CG31810-EGFP
3 pBac-CG6776-EGFP
4 pBac-Mdr50-EGFP
5 pBac[3×P3-EGFPamf]
6 pHA3PIG
M Marker

Figure 9: Result of* BamHI *restriction of promoter-EGFP pBac vectors (1 µl of a 1:10 mp dilution, 1% agarose gel). White arrows indicate the determinative fragments for correct GFP-reporter construct orientation.

3.3 Sequencing reactions

The DNA sequence resulting from the first sequencing reaction of EGFP in the PSL/Gal4 vector, which was carried out after the first cloning step, is exactly identical to the theoretical EGFP DNA sequence. Based on the results of the second sequencing reaction of EGFP as part of the GFP-reporter construct in the different pBac vectors an alignment was made (Appendix XXIV). All sequencing reactions were successful, except for the sequencing reaction conducted with the reverse primer pBacC10R in the pBac-CG31810-EGFP vector. Therefore the 5' end of the EGFP DNA sequence is missing. No mistakes or uncertainties are found in the sequencing result of EGFP in the pBac-CG31809-EGFP, pBac-Mdr50-EGFP, pBac-CG6776-EGFP and the 3' end of the pBac-CG31810-EGFP vector.
The sequencing results of the 3' end of the promoter and the 5' end of the terminator of all GFP-reporter constructs in the PSL-Gal4 vectors are very good, only primer PSLC76R did not give a result. A set of alignments was performed with the theoretical construct DNA sequence and the sequencing result of EGFP in the pBac vector, which did not reveal any alignment problems.
A comparison of the sequencing results of the gene specific promoters and terminators with the theoretical DNA sequences available at FlyBase (h*ttp://flybase.bio.indiana.edu)* revealed that the promoters of genes CG31809 and CG31810 are exactly homologous to the theoretical sequences. However, the promoters of genes CG6776 and Mdr50 showed some variation to the theoretical DNA sequence, respectively 2.6% and 3.5% changes in nucleotides (nucleotide replacements, insertions or deletions). This may represent the evolution of laboratory strains over time.

3.4 Microinjection experiments

The survival rate of the microinjected embryos and embryos subjected to the microinjection protocol without microinjection itself, were determined.
Table 3 gives an overview of the survival rates of embryos microinjected with the different pBac vectors (Appendix XXIII). Of the total of 3759 microinjected *Drosophila* embryos, 1216 larvae hatched, of which 813 developed to adult flies. This gives survival rates of 32% from embryo to larvae, 67% from larvae to fly and 22% from embryo to fly.
The survival rate of the embryos subjected to the microinjection protocol without microinjection itself, is given in Table 4 and is the average rate of 5 different experiments (Appendix XXIII). These experiments resulted in a larvae survival percentage from egg to larvae varying between 23% and 64%, with an average survival percentage of 46%.

Table 3: Survival rate of microinjected Drosophila embryos

CONSTRUCT	# EGGS (E)	# LARVAE (L)	% L	# FLIES (F)	% –E-F	% –L-F
pBac-CG31809-EGFP	872	225	26	131	15	58
pBac-CG31810-EGFP	966	335	35	222	23	66
pBac-Mdr50-EGFP	902	247	27	142	16	57
pBac-CG6776-EGFP	803	343	43	274	34	80
pBac[3×P3-ECFPamf]	216	66	31	44	20	67

T	**3759**	**1216**	**32%**	**813**	**22%**	**67%**

Table 4: Survival rate of* Drosophila *larvae subjected to the micro-injection protocol without performing microinjection itself.

CONSTRUCT	# EGGS (E)	# LARVAE (L)	% L
-	272	126	**46**

3.5 Fluorescence microscopy

In none of the potentially transformed G_1 larvae investigated ECFP fluorescence could be detected in the eyes or nerve system. In addition, none of the G_1 adult flies screened displayed ECFP fluorescence in the eyes.
EGFP fluorescence is observed in the potentially transformed G_1 larvae, especially in the intestines. However, this EGFP fluorescence was not significantly different compared to the background EGFP fluorescence displayed by non-injected control larvae (Figure 10 & 11).

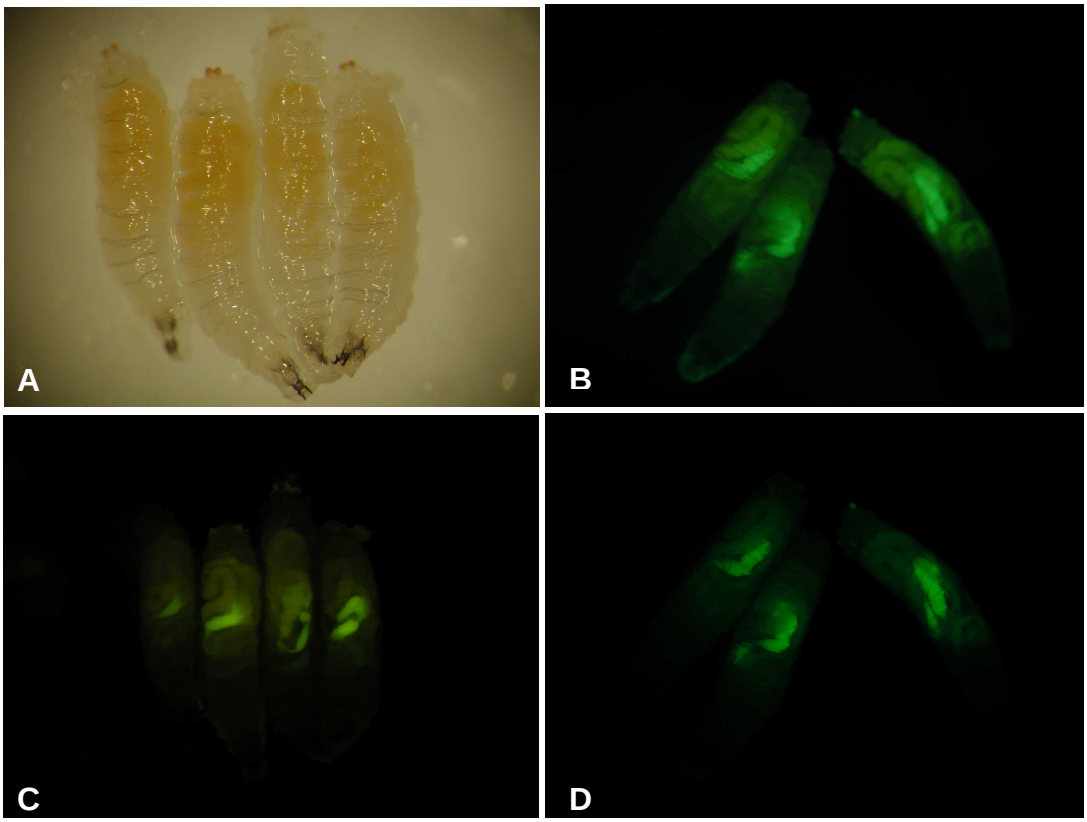


Figure 10: Non-injected* Drosophila *larvae observed with a Leica MZ FLII fluorescence stereomicroscope through filter sets: A) Bright-field B) Filter 1 C) Filter 2 D) Filter 3 (Photo's: D.M.A. van den Heuvel)

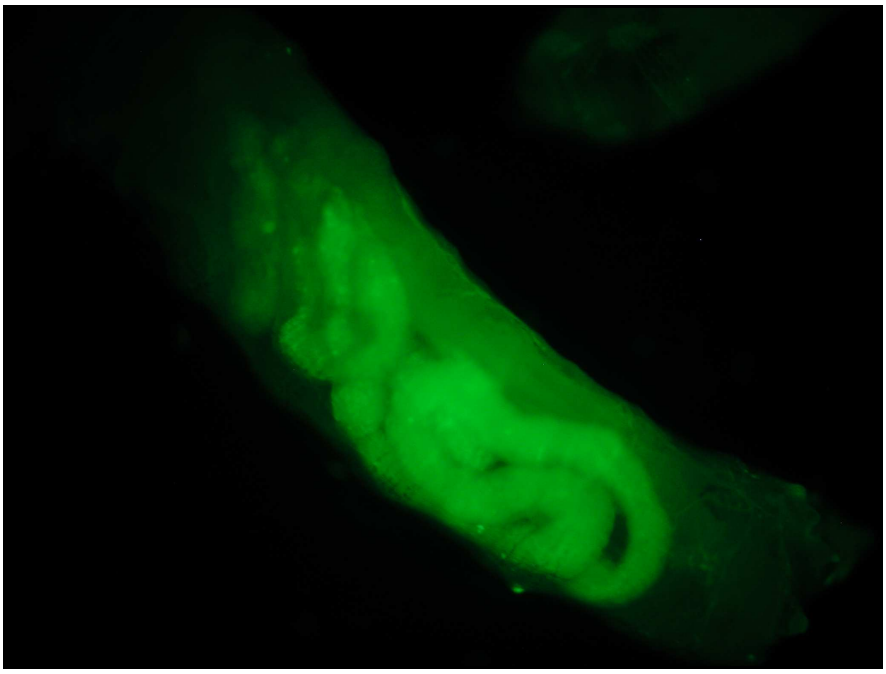


Figure 11: Close-up EGFP fluorescence in the intestines of non-injected* Drosophila *larvae observed with a Leica MZ FLII fluorescence steromicroscope through Filter 3. (Photo: D.M.A. van den Heuvel)

CH4 Discussion

4.1 Vector construction and microinjection survival rate

The investigation of the presence of antioxidant responsive element (ARE) enhancers in the promoter and terminator sequences of the selected genes pointed out that the ARE consensus sequence in its strict form: 5'- A/G TGA C/T NNNGC A/G - 3' (Hayes & McMahon, 2001) is not present. However, quite some potential ARE enhancer sequences, differing in 1 to 3 nucleotides from the consensus sequence could be identified. It is hypothesized that the promoter and terminator sequences of the selected genes do contain ARE enhancers, for the reason that in particular ARE enhancers are known to upregulate the expression level of genes which comprise the antioxidant system (Hayes & McMahon, 2001). In this respect, it is remarkable that the glutathione S-transferase gene CG6776, of which the expression of the whole gene family is known to be regulated by antioxidant promoting compounds interacting with ARE enhancers, is the only gene in this investigation in which not even potential ARE enhancer sequences could be identified. Further research on this subject is needed to reveal whether the potential ARE enhancers indicated in this research can be classified as real ARE enhancers or not.

The results of all endonuclease reactions mediated by the *EcoR*I, *XbaI* and *BamHI* enzymes are identical to the results expected in case of correctly constructed pBac vectors, except for two.

In the *EcoRI* restriction of pBac-CG31810-EGFP the expected 286 bp fragment did not show up (Figure 7). This can be explained by the relatively small size of the fragment which makes it invisible on gel. In the *BamHI* restriction of pBac-CG6776-EGFP the 1300 bp fragment is missing and additional bands showed up of: 1700bp, 2200 bp and 3000 bp (Figure 8). An explanation for this restriction result can not be given with certainty. It is probably caused by variation in the DNA sequence, the replacement, addition and removal of nucleotides, which results in the disappearance and formation of new restriction sites. In addition to this, the concentrations of the 1700 bp and 2200 bp bands are significantly lower than the concentration of the other restriction fragments. Such an observation points to the occurrence of partial digestion.

Of all EGFP sequencing reactions in the GFP-reporter pBac vectors one sequencing reaction failed, resulting in the absence of the EGFP 5' end in the pBac-CG31810-EGFP vector. Considering that there were no mistakes or uncertainties found in the sequencing results of the other pBac vectors it was assumed that the missing part of the EGFP sequence in the pBac-CG31810-EGFP vector was actually there and not further checked for sequence mistakes.

The survival rates (*Table 3*) of the microinjected and control embryos exposed to the same protocols were respectively 32% and 46%. This is regarded to be only a slight difference in survival and an indication of the quality of the injection procedure. This can be stated more convincingly by paying attention to the fact that these survival rates are average numbers based on the survival rates of all the different microinjection experiments executed (*Appendix X*), which vary to between 9% and 67%. This big variation in survival rate is thought to be caused by the relatively great influence of some particular experimental steps in the microinjection protocol on the survival rate. Experimental steps which are thought to strongly influence the survival of the embryos are the time period of embryo dechorionation with 50% bleach, the drying time of the dechorionated embryos on silica gel, insufficient coverage of the embryos with Voltalef oil and rough handling of the embryos in general.

The observation that on average one third of the larvae developed from the microinjected eggs will not make it to the flies stage (*Tabel 3*) is thought to be due to a less or greater extent by the microinjection

performed in the embryo stage. Microinjection is hypothesized to cause particular injury to the embryo, which can possibly turn out to be lethal during the metamorphosis stage from larvae to fly. However, an indication to which extent microinjection influences this survival rate can not be given based on these experimental results, because data on the survival of larvae to fly for embryos subjected to the mechanical microinjection protocol without fluid injection were not collected.
For the reason that for the separate microinjection experiments survival rates (from egg to larvae) up to 67% are observed, the microinjection protocol is thought to be highly efficient in case it is precisely executed. This is based on the fact that for untreated *Drosophila* embryos a maximum survival percentage of 70% is observed (pers com M. van Schie LUMC).

4.2 Germline transformation

Neither ECFP nor EGFP fluorescence could be identified in the potentially transformed *Drosophila* larvae and flies screened by fluorescence microscopy. With respect to this result it is most logical to conclude that none of the *Drosophila* larvae and flies examined were genetically transformed.
There are a lot of experimental aspects related to *Drosophila* germline transformation and the screening method which could potentially explain this negative result. The problem could be related to the DNA constructs, the helper vector and the screening method.

Despite elaborate testing by endonuclease and DNA sequencing reactions there still could be something wrong with the pBac constructs and/or helper vector. The functionality of the original pBac transformation vector, pBac[3×P3-ECFPamf] vector, was succesfully used in *Bombyx* transformations at PRI so that we do not expect the vector itself to be corrupt.
Another possibility is that there was something wrong with the pBac helper vector. In this research only a simple restriction reaction was performed to verify that the isolated vector was indeed the helper vector. However, vector multiplication in bacteria can bring along changes in the DNA sequence. There is a possibility that the *piggyBac* transposase coding sequence was changed, which resulted in the misexpression of the transposase protein. In case of a defect transposase protein *Drosophila* germline transformation is impossible. The quality of the GFP constructs could have been verified by taking advantage of transient expression in the G0 larvae and flies. In that case the G_0 larvae should have been screened for ECFP and EGFP expression in addition to the G_1 larvae which were screened in this research. In the G_0 larvae transient EGFP expression can be observed when the vector plasmid is transcribed and translated in the cytoplasm. When a certain number of G_0 larvae with ECFP and EGFP transient expression would have been observed, but never a single G_1 larvae expressing these fluorescent proteins would have been detected, it could have been concluded that the construct was working fine, but could not integrate in the genome with a frequency above some specified number.
However, apart from the possibility that transgenic lines were never generated, it is also possible that they were never detected. There are different experimental aspects which can cause a low and possibly undetectable expression of the fluorescent proteins. There is the possibility of the occurrence of a negative chromosomal position effect, where the specific position of construct integration in the genome can suppress its expression. In addition, it is known that in some transgenic insect lines certain aspects of the fluorescence pattern are not suppressed, which is most likely due to developmental stage- or tissue-specific position effects (Horn et al. 2000). This is for example the case in the research of Hediger et al. (2001) in which of 3×P3-EGFP pBac transformed larvae of the

housefly *Musca domestica* expressed fluorescence in the developing eyes only in a limited number of larvae

However, these two events are not likely to explain the absence of germline transformation in itself. They only help to explain the relatively low frequency of transformation which is usually recorded. Presently, *Drosophila* germline transformation by microinjection is a commonly used research technique worldwide and *piggyBac* vectors have shown to integrate with a high efficiency in the germline of different dipteran species (Handler & Harrell, 1999). Known from literature, researchers experience different *piggyBac* transformation efficiencies varying from 35% for Berghammer, et al. (1999), 2% for Horn et al. (2000), 1% for Handler & Harrell (1999) to 0.125% for Depra et al. (2004).

Besides the arguments stated above that try to explain the absence of ECFP and EGFP fluorescence in different ways, it could also be possible that the *Drosophila* screened were genetically transformed and indeed expressed the fluorescent ECFP and EGFP proteins, but that this could not be detected by fluorescence microscopy. It is hypothesized that with the available equipment and insect diets used the observation of ECFP and EGFP fluorescence in the *Drosophila* larvae by fluorescence microscopy was at least very difficult or even impossible.

Firstly, in the case of the detection of ECFP fluorescence the main problem has been that the excitation wavelength of ECFP causes significantly increased autofluorescence compared to the excitation wavelengths of other fluorescent proteins as for example EGFP (Horn et al., 2002). Secondly,, the GFP1 filter used to detect ECFP fluorescence is not a selective filter in the sense that not only fluorescence light emitted by ECFP is observed, also fluorescence light of other fluorescent proteins like EGFP and autofluorescence is transmitted. It is hypothesized that this combination makes it very difficult to discriminate the 3×ECFP expression pattern from the EGFP fluorescence and autofluorescence observed in the larvae.

Autofluorescence is definitely thought to be the most important reason for the inability to observe EGFP fluorescence. Uninjected *Drosophila* larvae emit a lot of autofluorescence from ingested food induced by the excitation light beam for EGFP fluorescence (Figures 10 & 11). In none of the injected larvae screened a significant higher level of ECFP and EGFP fluorescence could be detected compared to fluorescence observed in the uninjected larvae.

In this research it could have been verified whether autofluorescence and the use of unselective filter sets indeed made it impossible to observe the ECFP and EGFP fluorescent proteins. This could have been done by screening all *Drosophila* G_1 flies for ECFP expression in their compound eyes, which observation is not hindered by autofluorescence for the reason that ECFP is expressed at a very high level in all photoreceptor cells.

CH5 Conclusion

The *Drosophila* genes were selected for the construction of the GFP-reporter constructs, based on their 5-70 fold upregulation in response to induction with curcumin in the microarray studies. However none of these genes contained the mammalian antioxidant responsive element (ARE) enhancer in its strict form. However, in most gene promoters and even in the terminator sequences of the steroid dehydrogenase genes potential ARE enhancers could be identified, differing in 1 to 3 nucleotides from the ARE consensus sequence. The genuineness of these potential ARE enhancers would have to be demonstrated in further research.

A sequence homology search between the promoter and terminator sequences of the selected genes revealed that large parts of the promoters of the steroid dehydrogenase genes CG31809 and CG31810, close to the start of the coding sequence, are highly homologous.

The results of the different endonuclease and DNA sequencing reactions that were carried out demonstrate that all GFP-reporter construct pBac vectors have the correct error free DNA fragments incorporated in the correct orientation.

Fluorescence microscopy could not identify genetically transformed *Drosophila* expressing ECFP and EGFP fluorescence. This could be caused by the lack of transgenics in the set screened or by the inability to detect them with the current set up. We believe that the second explanation is most likely. The pBac construct vectors were demonstrated to be correctly constructed, and the procedure of embryo microinjection only slightly negatively affected the embryo survival rate. This suggests that we should have observed some positive larvae among the ……screened. The *piggyBac* transformation of *Drosophila* is a commonly used technique worldwide with good transformation efficiencies in general. It is hypothesized that there were genetically transformed *Drosophila* present among the screened larvae, expressing the fluorescent proteins, but that it was at least very difficult or even impossible to distinguish between the transformed and untransformed larvae by fluorescence microscopy. Arguments that support this hypothesis are that the excitation of ECFP causes a higher level of autofluorescence compared to the excitation of other fluorescent proteins and that the GFP1 filter used for the detection of ECFP fluorescence is not selective, which means that fluorescence of other fluorescent proteins like EGFP fluorescence is also transmitted (Horn et al., 2002). This together is thought to make it very difficult or even impossible to identify the 3×ECFP expression pattern from the EGFP fluorescence and autofluorescence observed in the larvae. In addition, the observation of EGFP fluorescence is thought to be hindered to a great extent by the high level of autofluorescence emitted from the food present in the larval intestines, induced by the excitation light beam resulting from the microscope filter sets used to detect EGFP. For the reason that the intestine tissue is the hypothesized place for the expression of the GFP-reporter constructs, observation of fluorescence emitted by the EGFP protein expressed from the transgenic constructs is considered to be impossible.

This research could not prove the suitability of a *Drosophila*-based screening assay for the identification of antioxidant compounds. However, it comes up with clear suggestions about what particular aspects of the initial concept of the assay have to be adjusted to solve the problems encountered in this research. Presuming that these problems can be solved, it is thought that the development of a *Drosophila*-based screening assay is a feasible goal and will lead to an efficient and reliable method to identify antioxidant compounds.

CH6 Recommendation

There are two important aspects of the experimental set-up of the *Drosophila*-based screening assay, for which improvement is essential for its feasibility.
In the first place, the fluorescence emitted from the different fluorescent proteins expressed from the reporter and marker construct should be completely distinguishable. This problem can not be solved for the ECFP/EGFP fluorescent protein combination, for the reason that there are no filter sets available that make their fluorescence emission completely distinguishable. However, there are a number of fluorescent protein combinations, which emissions can be unambiguously discerned by use of specific filter sets, even if they are expressed in the same tissue at the same time (Horn et al., 2002). This is the case for all fluorescent protein combinations which can be made of the trio ECFP, EYFP (yellowish mutant GFP variant; 514/527 nm) and DsRed (red fluorescing protein; 558/583 nm) and also for the EGFP and DsRed couple.
In the second place, autofluorescence from ingested food is interfering heavily. We tried several different foods but did not find a substrate without fluorescence. Nevertheless this may still be possible. Alternatively, the autofluorescence should be filtered from the fluorescence emitted by the protein expressed from the reporter construct. This can be solved by using a reporter construct based on the fluorescent protein DsRed. DsRed fluoresces outside the range of autofluorescence of most biological tissues, which results in a very high signal-to-noise ratio.
The DsRed fluorescent protein is isolated from a coral-like sea anemone of the genus *Discosoma* (Matz et al., 1999). It has about 23% amino acid sequence identity to GFP with conserved amino acids in the immediate vicinity of the fluorescence chromophore (Yarbrough et al., 2001). DsRed has shown to be highly resistant to photobleaching and to have a high quantum yield.
There are different variants of DsRed available. The pioneer protein DsRed1 is not suitable, because of its slow maturation time of approximately 24 h. The recently developed variant DsRed-Express (557 nm and 579 nm) is thought to be very well-suited candidate for the application as reporter protein in this research. It has a maturation time of 8-12 h which is comparable to that of EGFP. DsRed-Express has a lower quantum yield and extinction coefficient than DsRed1, however its improved folding efficiency more than makes up for the loss (Clontechniques, 2002).
In combination with DsRed-Express as reporter protein the application of the fluorescent EGFP protein as a marker protein is thought to be the best choice for the reason that a marker construct with EYFP is not available at PRI and ECFP induces a significant higher level of autofluorescence.
The selective filter that is needed to completely distinguish the fluorescence light emitted by DsRed-Express from the EGFP fluorescence is the Cy3.5/DsRed (436/20 nm, 480/40 nm) bandpass filter. EGFP fluorescence can be selectively observed with the YellowGFP (500/20 nm, 535/30 nm) bandpass filter. A disadvantage of the use of a bandpass filter, is that it has a low luminosity, which may only allow detection of medium to strong expression lines. However, this is not considered to be a problem in this research for the reason that strong expression of both the reporter and marker construct is expected after induction with curcumin or other antioxidant reaction inducing compounds.

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