Development of broadly applicable transgenic tools for the transposon mutagenesis of the red flour beetle, *Tribolium castaneum*

Dissertation

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To my parents & grandma Anna

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ABBREVIATIONS AND CONVENTIONS

3xP3	an artificial promoter containing three copies of P3 binding sites for dimers of Pax-6 transcription factor
ad	activation domain
bp	base pair(s)
β-gal	β-galactosidase
℃	grade(s) of Celsius
CNS	central nervous system
Df	chromosomal deficiency
DMSO	dimethyl sulfoxid
DNA	deoxyribonucleic acid
DNA-bd	DNA-binding domain
DsRed1	red fluorescence marker
DTT	dithiotreitol
E. coli	*Escherichia coli*
ECFP	an enhanced blue form of GFP
EGFP	an enhanced GFP
EYFP	an enhanced yellow form of GFP
F	farad, a unit of electric capacitance
FLP	flipase (recombinase) of yeast site-specific recombination system
FRT	FLP target site
GAD	Gal4 activation domain
Gal4	yeast transcriptional factor involved in galactose catabolism
Gal4VP16	DNA-binding domain of Gal4 fused to VP16
Gal4Δ	a deletion version of original yeast Gal4
GFP	green fluorescent protein
hsp70	heat-shock promoter of Hsp70 proteins
h	hour(s)
In	chromosomal inversion
kb	kilo base pairs (10^3 bp)
lacZ	gene encoding β-galactosidase in *E. coli*
LexA	a repressor protein of *E. coli*
$(LL)_4$	LL responder (four repeats) of LexA operator
mOD/min	10^{-3} optical density per minute
min	minute(s)

µl	microliter(s)
mRNA	messenger RNA
nls	nuclear localization sequence
Ω	ohm(s), unit of electrical resistance
ONP	yellow compound, o-nitrophenol
ONPG	uncoloured compound, o-nitrophenol- β-D-galactoside
PNS	peripheral nervous system
PUb	polyubiquitin promoter
RNA	ribonucleic acid
rpm	revolution(s) per minute
RT	room temperature
s	second(s)
SEM	scanning electron microscopy
scrtTA	single chain of reverted tTA
sctTA	single chain of tTA
SV40 polyA	polyadenylation signal of SV40 virus for mRNA processing
TE(s)	transposable element(s)
TetR	tetracycline repressor protein of *E. coli*
TIR	terminal inverted repeats
TRE	tetracycline-responsive element
Triton X-100	t-octylphenoxypolyethoxyethanol
tTA	DNA-binding domain of TetR fused to VP16
UASG	upstream activation sequence of GAL genes in yeast
UASp	upstream activating sequence in germline of *D. melanogaster*
UAST	upstream activating sequence in soma of *D. melanogaster*
V	volt(s)
VP16	activation domain of *Herpes simplex* virus
X-gal	a lactose analogue (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

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1 INTRODUCTION

The Coleoptera is the most successful order of insects. There are about 300,000 described species, representing a quarter of all catalogued inset species (Arnett 1967) and thus belonging to the most species-diverse eukaryotic order (Farrell 1998). Importantly, beetles are a far diverged group from Diptera (Kristensen 1999) and play a key role in comparative studies.

The red flour beetle, *Tribolium castaneum* (Coleoptera, *Tenebrionidae*) is an important pest in a wide variety of cereal products, especially in tropical and semi-tropical regions (Sokoloff 1972). Its economic relevance gathered entomologists to deal with this pest to try developing secure methods to bring its populations under control. Its easy and undemanding rearing on flour medium enriched by brewer's yeast introduced this species into laboratory, consequently attracting an attention of e.g. ecologist, physiologists and also geneticists already in mid of last century. By now, advanced methods such as *in situ* hybridizations (Brown et al. 1994a, 1994b), parental and/or embryonic RNA interference (Brown et al. 1999, Bucher et al. 2002) or germ-line transformation (Berghammer et al. 1999, Lorenzen et al. 2002) have been established in *Tribolium castaneum*, suggesting this species to be the key experimental specimen within the order Coleoptera. Moreover, recent progress in genomics and bioinformatics can tremendously contribute to understanding of *Tribolium* biological functions and will be applicable to other primitive non-dipteran insect species. The *Tribolium* genome has been sequenced and is available since the beginning of this year. This emphasizes the necessity to link the genomic sequence data to their biological functions.

Tribolium transgenesis was impossible until recently broad-range transposable elements, namely *Minos*, *Hermes*, and *piggyBac*, and universal transformation markers were developed (reviewed in Horn et al. 2002), which revolutionized insect transgenesis and enabled to overcome restrictions of genetic techniques to the model organism such as *Drosophila melanogaster* (Rubin and Spradling 1982, Spradling and Rubin 1982, Spradling et al. 1995). The preliminary tests revealed successful generation of transgenic *Tribolium* lines by using broad-range transposable elements (Berghammer et al. 1999, Pavlopoulos et al. 2004), precise excision/remobilization of *piggyBac* (Lorenzen et al. 2003) as well as functionality of discernable fluorescent markers (Berghammer et al. 1999, M. Klingler personal comm. and this thesis).

In regard to that, a novel and broadly applicable genetic tool for *piggyBac*-based insertional mutagenesis was developed and successfully tested in *D. melanogaster* (Horn et al. 2003) and its introduction into *T. castaneum* has been started (E. A. Wimmer, personal comm.). In combination with other versatile systems used in *D. melanogaster* genetics such as enhancer trapping (O'Kane and Gehring 1987), directed binary expression systems (Brand and Perrimon 1993, Bello et al. 1992, Szüts and Bienz 2000) or the site-specific recombination systems (Golic

and Lindquist 1998, Siegal and Hartl 1996), the most sophisticated system for genetic manipulation outside of *Drosophilidae* can now be established.

Design of such universal tools and their functionality in *T. castaneum* will be crucial for evolutionary developmental biology, semiochemical-behavioural biology, biotechnology and/or improvement of insect control techniques of agricultural-economically relevant pest species.

1.1 MOLECULAR BASIS OF INSECT TRANSGENESIS

Until recently, to create transgenic animals has been possible only in *Drosophila* species (Rubin and Spradling 1982, Spradling and Rubin 1982), caused by the fact that *P*-elements do not function outside of *Drosophilidae* (Handler et al. 1993). It took years to realize that entering other non-drosophilid species is possible by more promiscuous transposable elements from other insects (reviewed in Handler and James 2000).

The germ-line transformation of genetic model *Drosophila melanogaster* is routinely carried out by using a two component system, containing a 'DNA-construct' in *P* backbone and a 'helper-construct'. In principle, the latter construct can mobilize the first one and insert it anywhere in the *Drosophila* genome. This sometimes results in mutated genes and such 'DNA-constructs' can be called 'mutators'.

The identification of positive germ-line transformants is feasible by mutant rescue eye-colour selection in *Drosophila* species (Rubin and Spradling 1982) unlike in other insects, where investments to isolate the eye-specific genes is a time consuming effort, which needs to be carried out prior to new species transformation. A novel approach using a universal promoter, which drives visible eye-fluorescence or other visible phenotype distinct form the wild-type, would help to overcome that. Handler and Harrell (1999) showed that the enhanced variant of GFP protein, EGFP (Cormack et al. 1996; Yang et al. 1996) placed under the control of the constitutive *polyubiquitin* promoter enabled to identify new transgenic animals. The real revolution, however, caused an artificially multimerized 3xP3 promoter (Sheng et al. 1997) that is based on the transcriptional activator Pax-6/Eyeless (Callaerts et al., 1997). This reliably drove the fluorescent marker without disturbing autofluorescence in eye-specific manner in *Tribolium castaneum* and *Drosophila melanogaster* (Berghammer et al. 1999, Horn et al. 2000, Horn and Wimmer 2000). The divergence between beetles and flies being close to 300 million years (Kristensen 1999) suggested its potential functionality in all eye-bearing animals.

The eye-specifically expressed fluorescent colour serves to maintain transgenic stocks. In *Drosophila* genetics, mutants are kept by using balancer chromosomes, which carry dominant markers and recessive lethal mutations, so that only mutant heterozygots or alternatively mutant homozygots survive in the stock. As usually no balancer chromosomes exists for newly transformed insect species, eye-specific expression of fluorescent marker

allows to sort between non-transgenic homozygots and remaining transgenic animals, carrying the marker. Moreover, the use of separable fluorescent markers allows the independent identification and follow up of several distinct transgenic constructs in genetic crosses (reviewed in Horn et al. 2002).

1.2 TRANSPOSON-BASED INSERTIONAL MUTAGENESIS

An advanced system, containing besides 'mutator' another component, called 'jumpstarter', was developed to transpose 'mutator' throughout the genome of *D. melanogaster* (Cooley et al. 1988, Spradling et al. 1995). The principle originated from the '*PM* hybrid dysgenesis' phenomenon (Ashburner 1989), when females of cytotype *M* that were crossed to males of cytotype *P* caused *P*-element transposition in germ-line. It was suggested that *P*-element was horizontally transferred by parasitic mite from other species *D. wilistoni* within last sixty years (Houck et al. 1991).

In regard to the broad-range transposable elements, prior to their usage in new insect species a similar transposase source for mobilizing a 'mutator' should be considered. The principle of insertional mutagenesis by using other than *P*-elements requires that 'mutator' and 'jumpstarter' are based on two different types of transposable elements. Only on this condition 'jumpstarter' is stably integrated and can thus be efficiently removed allowing new insertions of 'mutator' to be stabilized. The crucial point is that both are non-autonomous so that the 'jumpstarter' encoding transposase can not cross mobilize the backbone it is embedded in. Importantly, both 'jumpstarter' and 'mutator' are marked by distinct markers to identify at the same time whether or not they are present in transgenic animals.

On condition that 'mutator' contains basal promoter, not only mutations of some genes in the genome will be obtained. The basal promoter can come under the control of some enhancer, i.e. *cis*-regulatory sequence, and cause expression change of 'mutator' fluorescent marker, a so called "enhancer trap". The neighbouring genomic DNA can be molecularly characterized by inverse PCR (Ochman et al. 1988). Interesting mutant phenotypes or enhancer traps, therefore, can be linked to their precise genomic position. This is a key advantage of transposon-based insertional mutagenesis.

Recently, novel and universal genetic tools based on 'mutator' in *piggyBac* backbone was developed and preliminarily tested in *D. melanogaster* (Horn et al. 2003). The *piggyBac* backbone displays several advantages: (i) its excision from the germline is almost always precise and (ii) does not suffer from preferential integration sites (hotspots) like *P*-elements (Spradling et al. 1999). Several mutant phenotypes and enhancer traps were identified. The evidence of its functionality in *D. melanogaster* paved the way for further introduction of this

universal tool into new non-drosophilid insect species as well as contributes to the increase of *Drosophila* genome coverage (Häcker et al. 2003, Thibault et al. 2004).

However, the relevant question is how to drive 'jumpstarter' elements in non-drosophilid species, such as *Tribolium castaneum*. Lorenzen et al. (2003) mediated *piggyBac* remobilization by injecting *piggyBac* transposase source into embryos of *T. castaneum,* suggesting that insertional mutagenesis screen is feasible in this species. Nonetheless, such approach was labour-intensive and more convenient transgenic lines with suitable promoter to conditionally provide transposase source were of need. In *D. melanogaster* either temperature sensitive promoters of the Hsp70 protein family (*hsp70* promoter, Lis et al. 1983) or constitutively driven *α1-tubulin* promoter (*α-tub*, Theurkauf et al. 1986) are mainly used. Recent works (Presnail and Hoy 1992, Uhlířová et al. 2000) pointed out that *Drosophila hsp70* promoter is also functional outside *Drosophila* species and therefore a potential candidate for *Tribolium* to transiently drive *piggyBac* transposase source *in vivo*. Therefore, detailed analyses of *Drosophila hsp70* was required to evaluate its functionality and potential suitability in transposon-based insertional mutagenesis screens of the red flour beetle, *Tribolium castaneum*.

1.2.1 Binary expression systems

Binary expression systems are widely used as genetic tools in *Drosophila melanogaster*. They are composed of transactivator and responder, which are inactive until they are brought together (Fig. 1-1). As several promoters and enhancer traps were identified in this species, spatially and temporally specific expression of any gene of one's interest can be performed there.

If such approach is applicable also in other species, it would greatly improve the functional analysis of genes. The transactivator of these systems can be included in 'mutator' elements and therefore distributed throughout the genome by insertional mutagenesis. Then not only mutations of novel genes will be obtained to study their function, but interesting *cis*-regulatory sequences (enhancer traps) can be used for miss-expression studies to drive: (i) reporter gene; (ii) any gene of one's interest, or (iii) RNAi to silence or knock-out particular genes. Such approach will be especially helpful for developmental, evolutionary and behavioural studies.

In *Drosophila melanogaster*, three binary expression systems are used: (i) Gal4/*UAS* system (Brand and Perrimon 1993); (ii) tetracycline-controlled system (Bello et al. 1998) and (iii) LexA/$(LL)_4$ (Szüts and Bienz 2000). Although they all work in this species, it is unknown which of the system works best and how they compare in their efficiency. Moreover, it will need to be tested, whether or not they will also function in *Tribolium castaneum*.

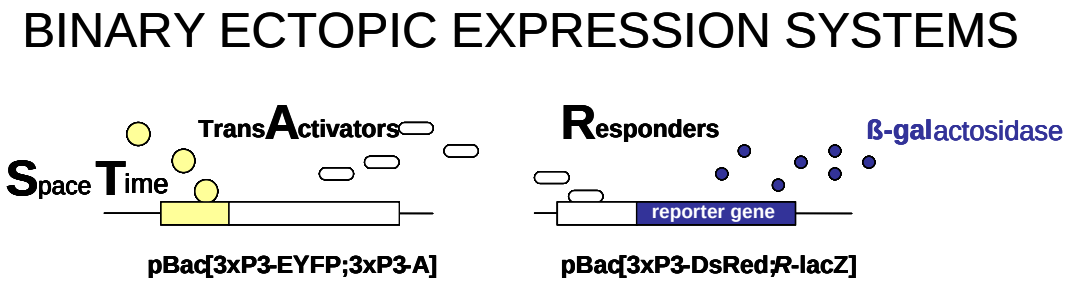


Figure 1-1 General scheme of binary expression system.
Spatial and temporal regulated transactivator (A, here under 3xP3 promoter) binds responder (R) sequence to drive the reporter gene, *lacZ*, expression. Both, A and R are fluorescently marked with yellow form of enhanced GFP (EYFP) and DsRed1, respectively, which are also placed under 3xP3 in *piggyBac* backbone (pBac). If both, A and R, are brought together, binary expression system is activated, reporter gene, *lacZ*, is driven and its product protein (β-gal) can be analyzed.

1.2.2 Chromosomal rearrangements

Random and time consuming chromosomal rearrangements by using physical, chemical or biological mutagens were replaced by yeast FLP/*FRT* recombination system, efficiently working in *Drosophila melanogaster* (Golic and Lindquist 1989). Since than it has become a powerful tool in *Drosophila* genetics. Although Cre/*loxP* was also introduced into this species (Siegal and Hartl 1996), its working efficiency has been referred as considerably lower than in plants (Qin et al. 1994) or mouse (Ramirez-Solis et al. 1995). The key advantage of these systems is that their recombinase acts specifically on its target sites, *FRT* or *loxP*, respectively. Therefore, designed chromosomal aberrations such as inversions, deletions or duplications can be created in the genome. This strongly contributed to *Drosophila* or mouse reverse genetic studies (Golic and Golic 1996, Zheng et al. 1999).

On the condition that 'mutator' element has incorporated such a recombination target site, this will be consequently distributed throughout the genome after proceeding with a transposon-based mutagenesis screen. Thus, several target sites will be available and various combinations can be used to create defined chromosomal rearrangements.

To be able to introduce a similar system to *Tribolium castaneum* and other insect species and use it *in vivo*, a novel and universal approach based on FLP/*FRT* site-specific recombination system (Golic and Lindquist 1989) was developed by Götschel (2003). Prior to its introduction into non-Drosophilids, preliminary tests are firstly required in the model organism, *D. melanogaster*.

1.2.2.1 Balancer chromosomes

The main purpose for generating chromosomal rearrangements comes from a necessity to establish balancer chromosomes in the red flour beetle, *T. castaneum*. Although recently a few balancer chromosomes have been created (Beeman 1986), to maintain several mutations

coming from the planned transposon-based mutagenesis screen requires to generate balancers to cover ideally the entire *Tribolium* genome. They enable to keep new stocks with a particular mutation and provide enormous advantages in rapid mapping of a particular mutation. When they are used in the mutagenesis screen (Hentges and Justice 2004): (i) the localization of particular mutation will be accelerated by either its exclusion or localization to the balanced inverted region; (ii) balanced stock will result in parallel; (iii) many homozygous embryonic-lethal phenotypes can be identified, which would normally die in embryonic stage and (vi) it allows to maintain quantitative or modifier traits besides single gene phenotypes.

Until recently, balancer chromosomes were more or less the domain of *Drosophila melanogaster* genetics. They were introduced to this species in 1918 by Muller, who identified that lethal mutations can be maintained -'balanced'-without a selection. Later on Sturtevant (1926) proposed that the reason for this 'balancing effect' was due to a comprised inversion between two lethal mutations, which were linked on the same chromosomal region *in trans*, i.e. each of them was on one homologous chromosome. Since then it has been known that inversions suppress meiotic recombination between inverted and its non-inverted, homologous chromosomal region. The chromosomal aberrations, resulting from such recombination, are selectively eliminated from the functional products of female meiosis in *D. melanogaster* and only normal non-recombinant chromatids are recovered. This is observed as suppression of the meiotic recombination.

Based on that, several balancer chromosomes were established and are nowadays routinely used as important genetic tools in *Drosophila* genetics (Ashburner 1989). They can be characterised as complex chromosomes with: (i) multiple inversions, suppressing meiotic recombination in females, to maintain stable stocks; (ii) a dominant selective marker, which makes balanced heterozygots visible as they usually affect adult or larval morphology (Ashburner 1989) or are tagged with fluorescent colour (Casso et al. 2000, Halfon et al. 2002), and (iii) recessive mutations, that cause lethality or reduced fecundity in balancer homozygots, so that no selection is required. The latter case is especially necessary in *Drosophila* or other insect populations, where random mating occurs and selection against balancer homozygots would be tedious or impossible.

However, the laborious development of such balancer chromosomes for *Drosophila* genetics (*X-rays*, *EMS* or *PM* dysgenesis) was time consuming due to difficult identification (e.g. polytene chromosome analyses or position-effect variegation). Although a few references to balancer chromosome exist in other species (Herman 1976, Forster et al. 1991, Hackstein et al. 1992, Beeman et al. 1986, Gourzi et al. 2000), the breakthrough in their establishment brought firstly the work of Zheng et al. (1999, 2001). The authors showed that defined inversions, which were created by using site-specific Cre/*loxP* recombination system in mouse (Ramirez-Solis et al. 1995), served as effective partial balancers. It inspired to develop and test a similar system

in genetic model *D. melanogaster* (Götschel 2003) based on the establishment of inversions by using FLP/*FRT* site-specific recombination (Golic and Golic 1996) to be later introduced outside the genus *Drosophila.*

1.2.2.2 Stabilization of transposable elements

Inversions are not only important to create balancer chromosomes. If *FRT* sites are inserted between left and right terminal inverted repeat (TIR) of used constructs, this inversion mediated by FLP/*FRT* site-specific recombination can result in reciprocal exchange of these TIRs. It was shown that the transposase source of *P*-element requires intact 5' and 3' ends (Mullins et al. 1989) and recombinant *P*-element flanked by two 3' TIRs is stabilized (Ryder et al. 2004).

The sequence differences of 5' and 3' TIRs suggested that even *piggyBac* transposase needs both TIRs (Elick et al.1997, Li et al. 2001). Recently, Handler et al. (2004) showed that introduction of head-to-tail tandem duplication of one of *piggyBac* TIRs and subsequent transposase-mediated excision of the internal duplicated TIR and non-duplicated one, results in stabilization of the remaining TIR. However, whether two 5' or two 3' TIRs of the *piggyBac* transposable element are sufficient for its transposase remained to be proven.

Importantly, other transposable element, *Hermes* (Warren et al. 1994), is frequently used besides *piggyBac* based constructs in insect transgenesis (Jasinskiene et al. 1998, Pinkerton et al. 2000) and is highly active in *D. melanogaster* (O'Brochta et al. 1995). It was observed that this element can be cross mobilized by *hAT* element, *hobo* (McGinnis et al. 1983, Sundararajan et al. 1999), which is also present in *D. melanogaster* strains. In regard to that, the question was raised whether also *Hermes* can be stabilized by removal of one TIR and be protected from potential remobilization in host species.

1.2.2.3 Deletions and duplications

The FLP/*FRT* site-specific recombination system in *D. melanogaster* is used to establish deletions and duplications (Golic and Golic 1996). This is important for *Drosophila* genetics, because: (i) particular genes of one's interest can be deleted; (ii) defined deletions can be used to identify modifiers of misexpression phenotype and to find, *de novo*, genes involved in biological processes or (iii) mutagenesis screens in deleted chromosomal background can be performed to easily identify homozygous lethal mutations.

Since the first deletion in *D. melanogaster* has been isolated (1914), until now, a collection of more than 5000 deletions is available in this species (FLYBASE 2003). Ryder et al. 2004 emphasized the necessity to isogenise the genetic background and precisely map

deletion's end points onto the completed *Drosophila* genome sequence (Adams et al. 2002). Based on that, a genetic and computational toolkit, the DrosDel isogenic kit (http://www.drosdel.org.uk), was developed to create defined deletions by using *P*-elements and the *FRT*-based approach to cover almost the entire *Drosophila* genome.

The importance of designed deletions in *D. melanogaster* suggests that a similar approach, which would be applicable in other non-drosophilid species, will enormously contribute to understand the biological function of sequence data of these species.

1.3 THE AIMS OF THIS THESIS

In order to transfer the developed *piggyBac*-based transposon mutagenesis system (Horn et al. 2003) to the red flour beetle *Tribolium castaneum*, I tested the *Drosophila hsp70* promoter (Lis et al. 1983) whether or not is suitable to drive *piggyBac* (Cary et al. 1989) transposase expression in the germline of the red flour beetle.

To establish the binary expression systems, Gal4/*UAS* (Brand and Perrimon 1993), the tetracycline-controlled system (Bello et al. 1992, Krueger et al. 2003) and LexA/*(LL)*$_4$ (Szüts and Bienz 2000) in the model organism *Drosophila melanogaster*, I designed systems, which: (i) are placed in *piggyBac* backbone; (ii) are marked with enhanced yellow form of GFP, EYFP (Cubitt et al. 1999) or DsRed1 (Handler and Harrell 2001) fluorescent protein under the control of the artificial 3xP3 promoter (Sheng et al. 1997) and (iii) have transactivators driven by 3xP3 promoter, so that identification of the reporter gene, *lacZ*, should be possible in the eyes. These systems will be firstly tested in the fruitfly for functionality, prior to their introduction into *T. castaneum*.

In addition, I analyzed the following defined chromosomal rearrangements (e.g. Rong and Golic in Handler and James 2000): (i) inversions to test their properties as partial balancer chromosomes; (ii) inversions to address the potential of *piggyBac* (Cary et al. 1989) and *Hermes* (Warren et al. 1994) stabilization, which is based on their rearranged TIRs; (iii) deletions/duplications to test how efficiently they can be established. They all will be created by using novel, broadly-applicable tools and tested in the model organism *D. melanogaster* (Götschel 2003). This tools comprise of *piggyBac* and *Hermes* based constructs, which are marked with yellow form of GFP, EYFP; blue form of GFP, ECFP (Patterson et al. 2001) and DsRed1 under the control either the artificial 3xP3 promoter or constitutively active *polyubiquitin* promoter, PUb (Harrell and Handler 1999). Each construct contains a *FRT* target site of the yeast FLP/*FRT* site-specific recombination system (Golic and Lindquist 1989) and due to the insertion of *FRT* sites into the 5' UTRs, promoter and fluorescent marker are separated upon recombination.

2 MATERIAL AND METHODS

2.1 MOLECULAR BIOLOGY

2.1.1 Standard methods

Molecular biology procedures and solutions followed protocols according to Sambrook et al. (1989), if it is not described differently in the following. Restriction enzymes were provided by Roche or New England Biolabs (NEB) companies. To blunt ends after restriction reaction, DNA polymerase I large (Klenow) fragment (Roche) was used. To ligate DNA fragments, T4 DNA ligase (NEB) or Fast-Link DNA Ligation Kit (Biozym) was utilized. Dephosphorylation of 5' phosphate groups of DNA was done by using alcaline phosphatase (CIP, SAP) that were provided by Roche. Experimental conditions were carried out according to manufacturers' protocols. To remove buffer salts from restriction reactions, spin dialysis was applied (described e.g. in Götschel 2003). To prepare plasmid DNA, either 'lazy lysis' (e.g. Götschel 2003) or Qia Miniprep Kit (Qiagen) were used. Qia Midiprep Kit (Qiagen) was used for final DNA plasmid preparation. To isolate DNA fragments from agarose gels, Qiaex II Gel Elution Kit (Qiagen) or NucleoSpin Extract (Macherey-Nagel) were used. The transformation of plasmid constructs was done by the heat-shock of *Escherichia coli* strain HB101, DH5α or the electroporation of its DH10β strain (Biorad, 25 µF puls, 2.5 kV, 200Ω). Primer syntheses and sequencing procedures were done by company, Medigenomix, Göttingen.

2.1.2 Cloned constructs

2.1.2.1 Prerequisite constructs

pSLfa_3xP3_fa: a 240 bp *Eco*RI-*Sal*I fragment of pSL-3xP3 (Horn et al. 2000), containing three copies of P3 and the TATA-homology, was cloned into *Eco*RI and *Sal*I digested pSLfa1180fa (Horn and Wimmer 2000).

pKS-LL: a 120 bp *Xba*I-*Asp*718 fragment of HZ50PL (Szüts and Bienz 2000) was cloned into *Xba*I and *Asp*718 digested pBluescript® II KS (Stratagene, Amsterdam).

2.1.2.2 Middle-step constructs

Transactivators

pSLfa_3xP3Gal4VP16_fa: a 960 bp *Bam*HI (Klenow blunted)-*Not*I fragment of pCSGal4/VP16 (provided by Köster 1999), containing original Gal4 DNA-bd fused to VP16 ad of *Herpes simplex* virus and SV40 polyA was cloned into the multiple cloning sites of *pSLfa_3xP3_fa* by its *Sal*I (Klenow blunted) and *Not*I digest.

pSLfa_3xP3Gal4Δ_fa: a 1150 bp *Asp*718-*Xba*I fragment of G610 plasmid (G. Struhl; G610 plasmid originated from the deletion variant II-9, Gal4Δ, made in the group of Ma and Ptashne 1987) was cloned into *Asp*718 and *Xba*I digested *pSLfa_3xP3Gal4VP16_fa* to replace Gal4 DNA-bd and VP16 ad by Gal4Δ variant of the original Gal4.

pSLfa_3xP3tetR2xVP16_fa: a 1 kb *Eco*RI (Klenow blunted) and *Bam*HI fragment of pTet-Off (Clontech, Palo Alto, USA) containing DNA-bd of tetR fused to VP16 ad (tTA) was cloned into *Asp*718 (Klenow blunted) and *Bgl*II digested *pSLfa_3xP3Gal4VP16_fa*. Because only Gal4 DNA-bd of *pSLfa_3xP3Gal4VP16_fa* was replaced by tTA, two VP16 ad were obtained.

pSLfa_3xP3tetRVP16_fa: a 1250 *Eco*RI-*Xho*I fragment of *pSLfa_3xP3tetR2xVP16_fa* contained 3xP3 driven DNA-bd of tetR fused to one VP16 ad and was cloned into *Eco*RI and *Xba*I digested pSLfa_hs43lacZ_fa (Pogoda 2001) to get SV40 polyA.

pSLfa_3xP3sctTA_fa: a 1480 bp *Eco*RI (Klenow blunt)-*Sal*I fragment of pWHE130(sB+sB), which was provided by Ch. Berens (Erlangen), and contained two tetRs as a monomer (single chain, sctetR), was cloned into *Asp*718 and *Sal*I digested *pSLfa_3xP3Gal4VP16_fa*. By this cloning step, Gal4 DNA-bd of *pSLfa_3xP3Gal4VP16_fa* was replaced by sctetR, which resulted in sctetR fused to VP16 ad (sctTA).

pSLfa_3xP3scrtTA_fa: a 1480 bp *Eco*RI (Klenow blunt)-*Sal*I fragment of pWHE130(sM2+sM2), which was provided by Ch. Berens (Microbiology, Erlangen), and contained two reverted versions of tetR as a monomer (single chain, scrtetR), was cloned into *Asp*718 and *Sal*I digested *pSLfa_3xP3Gal4VP16_fa*. Gal4 DNA-binding domain of *pSLfa_3xP3Gal4VP16_fa* was replaced by scrtetR resulting in scrtetR fused to VP16 ad (scrtTA).

pSLfa_3xP3lexAGal4_fa: a 1130 bp *Eco*RI-*Asp*718 (both Klenow blunted) fragment of pLF1 (Szüts) containing full-length of LexA linked to Gal4 ad (GAD) together with an efficient translational initiation context plus *nls* (LexAGAD, Szüts and Bienz 2000) was cloned into

*Asp*718 and *Xba*I digested *pSLfa_3xP3Gal4VP16_fa*. Gal4 DNA-bd and VP16 ad of *pSLfa_3xP3Gal4VP16_fa* were replaced by LexAGAD.

Responders

pSLfa_LL-lacZ_fa: a 120 bp *Xba*I-*Xho*I fragment of *pSK-LL*, containing four times multimerized *LL* responder sequence of bacterial LexA repressor protein, was cloned into multiple cloning sites of pSLfa_hs43lacZ_fa by its *Nhe*I and *Xho*I digest (Pogoda 2001).

2.1.2.3 Final constructs

In all following constructs, the 3xP3 driven transactivators or *lacZ* reporter gene placed under the responder sequence *$(LL)_4$* or *UAST*, were cloned into *piggyBac* backbone in the same transcriptional orientation as the 3xP3 driven transformation markers, EYFP or DsRed, respectively.

Transactivators

pBac[3xP3-EYFP;3xP3-Gal4VP16]: *Asc*I fragment of *pSLfa_3xP3Gal4VP16_fa* was cloned into *Asc*I digested *pBac[3xP3-EYFPafm]* (Horn and Wimmer 2000).

pBac[3xP3-EYFP;3xP3-Gal4Δ]: *Asc*I fragment of *pSLfa_3xP3Gal4Δ_fa* was cloned into *Asc*I digested *pBac[3xP3-EYFPafm]* (Horn and Wimmer 2000).

pBac[3xP3-EYFP;3xP3-tTA]: *Asc*I fragment of *pSLfa_3xP3tetRVP16_fa* was cloned into *Asc*I digested *pBac[3xP3-EYFPafm]* (Horn and Wimmer 2000).

pBac[3xP3-EYFP;3xP3-sctTA]: *Asc*I fragment of *pSLfa_3xP3sctTA_fa* was cloned into *Asc*I digested *pBac[3xP3-EYFPafm]* (Horn and Wimmer 2000).

pBac[3xP3-EYFP;3xP3-scrtTA]: *Asc*I fragment of *pSLfa_3xP3scrtTA_fa* was cloned into *Asc*I digested *pBac[3xP3-EYFPafm]* (Horn and Wimmer 2000).

pBac[3xP3-EYFP;3xP3-LexAGAD]: *Asc*I fragment of *pSLfa_3xP3lexAGal4_fa* was cloned into *Asc*I digested *pBac[3xP3-EYFPafm]* (Horn and Wimmer 2000).

Responders

pBac[3xP3-DsRed;(LL)$_4$-lacZ]: *Asc*I fragment of *pSLfa_LL-lacZ_fa* was cloned into *Asc*I digested *pBac[3xP3-DsRedaf]* (Horn et al. 2002).

pBac[3xP3-DsRed;UAST-lacZ]: *Asc*I fragment of pBac[3xP3-EYFP;*UAST*-lacZ], which was provided from M. Klingler (Erlangen) and contained *UAST* (Brand and Perrimon 1993) linked to lacZ-SV40 from pCaSpeR AUG β-gal, was cloned into *Asc*I digested *pBac[3xP3-DsRedaf]* (Horn et al. 2002).

2.2 ANIMAL BREEDING, STOCK KEEPING, GENETICS

2.2.1 *Drosophila melanogaster*

Standard procedures were followed (Roberts 1998, Greenspan 1997). In regard to *Drosophila* transgenic line nomenclature, 'M' corresponds to line with male origin while 'F' corresponds to female origin. The suffix at these letters means the number of *Drosophila* chromosome (.II, .III, .X). Balancer chromosomes (CyO, TM2, TM3, TM6, FM7) and Sb marker are described in Lindsley and Zimm (1992).

2.2.2 *Tribolium castaneum*

Beetles were reared and kept in 26℃ or 33℃ incub ators under standard conditions as described by Berghammer et al. (1999) and Lorenzen et al. (2003). Independent lines were signed with letters. Balancer chromosomes were not available, so that transgenic beetle stocks were controlled every generation for their transformation markers.

2.3 GERM-LINE TRANSFORMATION

2.3.1 *Drosophila melanogaster*

The transformation followed the standard procedure into w^- preblastoderm embryos of *D. melanogaster* according to Rubin and Spradling (1982) by using *piggyBac* constructs (see part 2.1.2.3), which was dissolved (500 ng/µl) in the injection buffer (5 mM KCl, 0.1 mM KH_2PO_4/Na_2HPO_4 pH 6.8) together with helper plasmid (300 ng/µl) as a *piggyBac* source (*phsp*-pBac, Handler and Harrell 1999). Femto Jet (Eppendorf) device with purchased needles

(Femtotips II, Eppendorf) were used for the injection procedure. Injected embryos were covered with halocarbon oil (Voltalef 10S, Lehmann & Voss, Hamburg). Larvae were separately collected and adult flies crossed against *w*$^{-}$. Transgenic flies with the fluorescent transformation marker were mapped for a chromosomal position and balanced over CyO (the 2nd chromosome), TM2 (the 3rd chromosome) or FM7 (*X* chromosome) balancers.

2.3.2 *Tribolium castaneum*

The homozygous white-eyed *T. castaneum* strain (*vermilion*^{white} mutant) was used. Embryos were collected within not more than 2-3$^{1/2}$ hours after oviposition in 26℃ incubator. They were washed with 2% bleach and rinsed with water (26℃ room temperature). After lining up onto the cover slips, embryos were injected mediolaterally with the mix of 500ng/µl *piggyBac* constructs and 300 ng/µl helper plasmid (see 2.3.1). No colour-solution for visualization was added. FemtoJet device and sterile original needles were used (Femtotips I and II, Eppendorf). Injections were completed within 6$^{1/2}$ hours after the oviposition. To provide humid conditions important for early development, injected embryos were placed into apple-agar juice plates in closed box (33℃ incubator). After two days, box li d was opened. Single hatched larvae were collected 3rd and 4th day after injection and let develop on whole grain flour that was enriched with 5% yeast (33℃ incubator). G_0 eclosed pupae were sorted for their gender and crossed against *vermilion*^{white} mutants with a correspondent gender. In G_1 generation, transgenic pupae or beetles were selected according to the transformation marker. Single G1 transgenic pupae were crossed together and G_2 progeny was tested for a single or multiple insertions.

2.4 TRANSFORMATION MARKERS, EPIFLUORESCENCE MICROSCOPY, DOCUMENTATION

Besides EGFP fluorescent marker (Cormack et al. 1996, Yang et al. 1996), the blue GFP variant, ECFP (Patterson et al. 2001) and the yellow GFP form, EYFP (Cubitt et al. 1999) as well as humanized variant DsRed1 (Handler and Harrell 2001). According to used promoter, PUb or 3xP3, fluorescent markers were ubiquitously expressed in *D. melanogaster* or observed in the eyes of *D. melanogaster* and *T. castaneum*.

To observe fluorescence markers, Leica MZ FLIII fluorescence stereomicroscope was used with planachromatic 0.5x or planapochromatic 1.6x objective. Different filter sets were used with a dependence on the nature of the fluorescence marker. The filter system consists of excitation and emission filters. To excite the light, mercury lamp was used. GFP2 longpass emission filter (GFP plus; Leica, Bensheim) allowing emitted light pass through above defined wavelength was utilized. To restrict the emitted light into defined spectral width, bandpass filters, yellowGFP (Chroma 41028; AHF analysentechnik AG, Tübingen), CyanGFP (Chroma

31044v2; AHF analysentechnik AG, Tübingen) and Cy3.5/DsRed (Chroma 41021; AHF analysentechnik AG, Tübingen) were used. The overview of filter systems is shown in Tab. 2-1.

Table 2-1 Filter systems and their spectral characteristic

Filter system	Excitation filter (λ_{max}/spectral width)	Emission filter (λ_{min} or λ_{max}/spectral width)	Fluorescence marker
GFP2 (GFP plus)	480 nm/40 nm	510 nm	EGFP, EYFP (DsRed1 transmits)
YellowGFP	500 nm/20 nm	535 nm/30 nm	EYFP
CyanGFP	436 nm/20 nm	480 nm/40 nm	ECFP
Cy3.5/DsRed	565 nm/30 nm	620 nm/60 nm	DsRed1

Photos were taken with AxioCam HR (Zeiss) by using Zeiss AxioVision 3.1 program. To immobilize larvae, pupae and adults, they were placed into 1.5 ml tube with Ringer's solution (Ashburner 1989) and heated for 5 min. in 65℃ heat-block and afterwards directly photographed.

Polytene chromosomes were analyzed with a Zeiss Axioplan 2 Imaging microscope and pictures taken by using Zeiss AxioVision program.

2.5 HEAT-SHOCK EXPERIMENTS

To test *Drosophila hsp70* promoter for its applicability in other species than *Drosophila*, transgenic lines containing *pBac[3xP3-DsRed, hsp70-EGFP]* construct were used. The *Drosophila hsp70* promoter originated from pCaSpeR-hs vector (Thummel and Pirrotta 1992). Construct and flies were provided by B. Jaunich, A. Pienimäki and E. A. Wimmer.

2.5.1 Heat-shock treatment in *Drosophila melanogaster*

The 3rd larval instar, pupae and adult flies were placed for 2 h into 37℃ incubator and then replaced into 25℃ room for 24 h. After this period, flies were anesthetized under CO_2 and dissected in Ringer's solution (Ashburner 1989).

2.5.2 Heat-shock treatment in *Tribolium castaneum*

Larvae, pupae and adult beetles were taken out of the flour food and put into new empty vials. These vials were kept for 1 h in 47℃ incubator. Afterwards, all developmental stages were replaced into vials with fresh food and kept in 25℃ incubator for 24 h to recover. Pupae were dissected in Ringer's solution.

2.6 BINARY EXPRESSION SYSTEMS ANALYSES

2.6.1 Analyzed constructs

Two components, the transactivator and the responder, of all analyzed system variants were placed into the *piggyBac* backbone. The universal 3xP3 promoter was used to drive all transactivators. As the reporter served ß-gal, this was placed under the responder activating sequence. To identify transgenic flies, fluorescent markers were cloned under the universal 3xP3 promoter in all constructs. To distinguish between two components of the system, two fluorescent markers were used: (i) enhanced yellow form of GFP (EYFP) for the transactivator and (ii) DsRed1 for the responder. Overview of analyzed constructs is shown in Tab. 2-2 (see also part 2.1.2.3).

Table 2-2 Analyzed constructs of the binary expression systems

System	Transactivator	Responder
Gal4/*UAS*	pBac[3xP3-EYFP;3xP3-Gal4]* pBac[3xP3-EYFP;3xP3-Gal4Δ] pBac[3xP3-EYFP;3xP3-Gal4VP16]	pBac[3xP3-DsRed;*UAST*-lacZ] pBac[3xP3-DsRed;*UASp*-lacZ]**
LexA/$(LL)_4$	pBac[3xP3-EYFP;3xP3-LexAGAD]	pBac[3xP3-DsRed;$(LL)_4$-lacZ]
Tetracycline-controlled system	pBac[3xP3-EYFP;3xP3-tTA] pBac[3xP3-EYFP;3xP3-sctTA] pBac[3xP3-EYFP;3xP3-scrtTA]	pBac[3xP3-DsRed;*TRE*-lacZ]**

* = the construct was made in the lab of M. Klingler; ** = constructs were made by C. Horn.

2.6.2 Kinetic analysis of the bacterial enzyme, β-galactosidase

To detect enzymatic activity of β-galactosidase in final extracts, which were prepared from heads of *D. melanogaster* and *T. castaneum*, o-nitrophenol-β-D-galactoside (ONPG) substrate was used

2.6.2.1 Analyzed lines

In *D. melanogaster*, three independent lines for each transactivator construct and each responder construct were used for kinetic analyses. The transactivator was signed as a letter 'A' and the responder as a letter 'R'. To distinguish independent lines, numbers were added as a suffix to 'A' or 'R' letters (Tab. 2-3). System variants were recognized according to a corresponding prefix name (e.g. Gal4-A1 or *UAST*-R1).

In *T. castaneum*, Gal4VP16 (line A) or Gal4Δ (line A3) transactivator constructs were combined with *UAST* (line B2) responder construct. Transgenic lines will be presented in results.

Table 2-3 Independent lines of transactivators and responders used for analyses in *D. melanogaster*

System	Transactivator		Responder	
	Name of Independent line	Line on the II. III. or X. chromosome	Name of independent line	Line on the II. or III. chromosome
Gal4/*UAS*	Gal4-A1	M1A.II	*UAST*-R1	M4.II
	Gal4-A2	M1B.II	*UAST*-R2	M5.III
	Gal4-A3	M1D.III	*UAST*-R3	M6.III
	Gal4Δ-A1	M1.III	*UASp*-R1	M2.II
	Gal4Δ-A2	M2.III	*UASp*-R2	M4.II
	Gal4Δ-A3	M9.II	*UASp*-R3	M5.III
	Gal4VP16-A1	F.II		
	Gal4VP16-A2	M.II-jump		
	Gal4VP16-A3	M.III-jump		
LexAGAD/$(LL)_4$	LexA-A1	F1C.III	$(LL)_4$-R1	M1.III
	LexA-A2	M4A.III	$(LL)_4$-R2	M14.III
	LexA-A3	F2B.X	$(LL)_4$-R3	M17D.II
Tetracycline-controlled system	tTA-A1	M2.III	*TRE*-R1	M3.II
	tTA-A2	M3.II	*TRE*-R2	M5.III
	tTA-A3	M10.II	*TRE*-R3	M6.II
	sctTA-A1	M3.III		
	sctTA-A2	M4.II		
	sctTA-A3	M9.II		
	scrtTA-A1	M2.II		
	scrtTA-A2	M4.II		
	scrtTA-A3	M5.X		

Three independent lines were chosen for each system variant. At least one out of three independent lines had construct insertion on different chromosome than the other two lines. jump = transgenic lines generated by construct remobilization.

2.6.2.2 Animal crosses

To activate the binary expression system, both components (i.e. the transactivator and the responder) were brought together. Animals containing the transactivator construct (3xP3-EYFP) were crossed against those that contained the responder construct (3xP3-DsRed1). The progeny was selected for both transformation markers and used for analyses.

In the case of *D. melanogaster*, two males of the transactivator line were crossed against at least two virgins of the responder line (or vice versa for *X*-linked transactivator line LexA-A3). Three independent lines were used for both, the transactivator and the responder, so that 3^2 different combinations were created for one type of the transactivator line that was combined with one type of the responder line. As a negative control served w^- flies and flies, which contained only the responder construct. The latter were the progeny of two w^- males, which were crossed against at least two virgins of the particular responder line. Fly crosses were kept

on the standard food with yeast at 25˚C incubator for 5 days, and then placed for another 5 days into 25˚C room till flies eclosed. Afterwards, 24 h old flies were collected and left at 25˚C room for 6 ½ -7 days. In the case of LexA/*(LL)*$_4$ system, flies were collected 10 h, 21 h and 24 h after their eclosure. They were also kept different time in 25℃ (6 and 8 days) and 18℃ room (27 days). To ensure about presence of both constructs, flies were checked for fluorescent markers before analysis.

In *T. castaneum*, male pupae containing the transactivator construct (3xP3-EYFP) were crossed against female pupae containing the responder construct (3xP3-DsRed1). They were fed on 5% yeast enriched whole grain flour with 0.03% Fumidil B in 33˚C incubator. Their progeny was checked for both constructs and kept for at least 10 days in 33˚C incubator. As a negative control served *vermilion*^{white} beetles and those, which contained only the responder construct. The latter were the progeny of *vermilion*^{white} male pupae that were crossed against female pupae of the particular responder line.

2.6.2.3 Detection of the reporter gene expression

Three male and three female heads of one cross were used to prepare one homogenate. As proposed by Jung et al. (2001), this head number was chosen to obviate a mistake of a small sample based on the data in *D. melanogaster*. Three independent homogenates were prepared, i.e. 18 heads per one cross were analyzed. To detect β-gal activity, ONPG substrate (Calbiochem®) was used. This has no colour until its cleavage by β-gal, resulting in yellow ONP product. The ONP was measured in final extracts, which were in 96-well microplate (Nunc-Immuno^{TM} Plate, MaxiSorp^{TM} Surface, *NUNC*^{TM}), at 410 nm wave length for 90 minutes in one minute interval. The measurements were carried out by using the µQuant, Universal Microplate Spetrophotometer (Bio-Tek instruments ®, INC.).

Enzyme buffer

0.2 mM sodium phosphate buffer pH 7.0
1 mM $MgCl_2$
10 mM DTT
100 mM NaCl

ONPG
0.15 g dissolved in 20 ml 250 mM sodium phosphate buffer pH 7.0 + 30 ml ddH_2O

Protocol

1) Prepare fresh enzyme buffer (60 µl/six heads) and pipette into 1.5 ml tubes.
2) Pre-cool tubes with buffer on ice, cut insect heads and put then into tubes.
3) Homogenate properly heads in buffer with purchased homogenizer (Biozym).
4) Spin samples at 14,000 rpm/ 10 min/ 4˚C.
5) Take 50 µl supernatant and place into new 1.5 ml tube (keep on ice!).
   Note: do not freeze extracts before analysis to alleviate a loss of the enzymatic activity!
6) Pipette 45 µl of this supernatant (final extract) into 96-well microplate just before the kinetic reading (see plate schema in Fig. 2-1).
7) Add 150 µl ONPG substrate with eight-tip micropipette.
8) Let read by microplate spectrophotometer at 410 nm for 90 min with 1 min interval at RT.

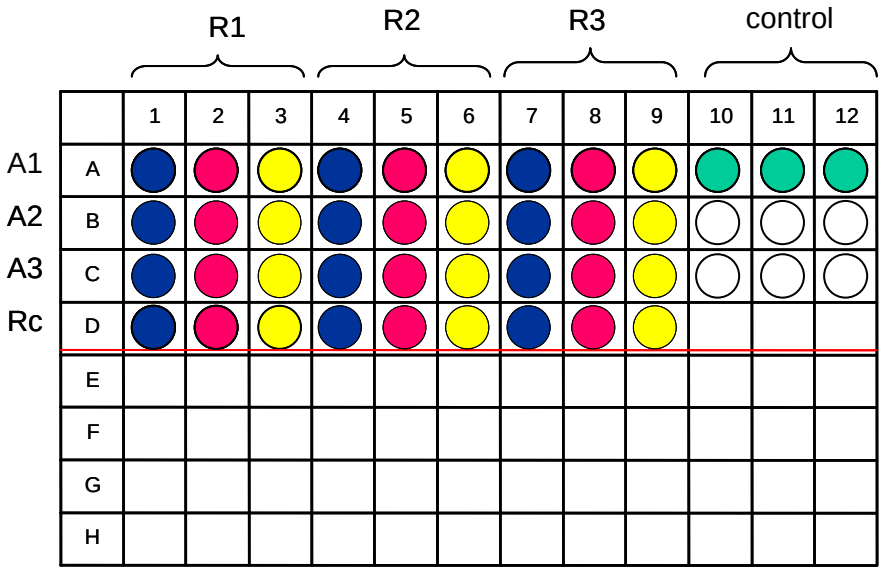


Figure 2-1 The 96-well microplate scheme.
A = a transactivator line; R = a responder line; Rc = a responder control, i.e. w^- males crossed against responder virgins. Each A line is combined with three independent responder lines and vice versa. Three independent extracts (blue, red, yellow coloured circles) were analyzed for each AxRx combination. w^- controls are green coloured. Enzyme buffer controls and ONPG controls are depicted as non-colour circles. Under red line other system was analysed in the same schematic way.

2.6.2.4 Evaluation of kinetic data

KC4 data reduction software was applied (version # 2.7, Bio-Tek instruments ®, INC.). KC4 data were reloaded into Microsoft Excel. The β-gal activity was then calculated as a subtraction of a reached optical density (OD) value at 410 nm in 90th minute and the OD value in the first minute of the kinetic reading. This value is expressed as mOD/min and corresponds to the enzymatic activity within 90 minutes. It is plotted as a line in the graph. Three

independent samples of one transactivator line (e.g. A1) that was combined with one responder line (e.g. R1) were analyzed (blue, red, yellow circles indicated in Fig. 2-1), which resulted in three reproducible lines in the graph (Fig. 2-2). The slope of one particular responder line varied for different transactivator lines (Fig. 2-3). OD range of responder control is shown in Fig. 2-4. The graph of w^- control was usually in the same or under OD range of the responder controls.

mOD/min was calculated over three samples (Fig. 2-2) of one transactivator/responder combination. Because there were three independent transactivator lines combined with three independent responder lines (Fig. 2-1), i.e. 3^2 possible combinations, average mOD/min was estimated over all those. It corresponded to combination of one transactivator variant, e.g. Gal4, which was combined with one responder variant, e.g. *UAST*. These nine combinations were analyzed two times; therefore, two average mOD/min values were estimated and used to assess mean mOD/min. This was used as final mOD/min value, which represented one particular system variant combination, e.g. Gal4/*UAST*. Responder controls were calculated over three samples of one responder line (e.g. R1 of $(LL)_4$ responder) as was shown in Fig. 2-4, then over three independent responder lines of one type (R1, R2, R3) corresponding to average mOD/min value for this responder type. Average mOD/min value of w^- control was based on three analyzed samples and then averaged over all obtained w^- controls, which corresponded to mean mOD/min value of w^- control.

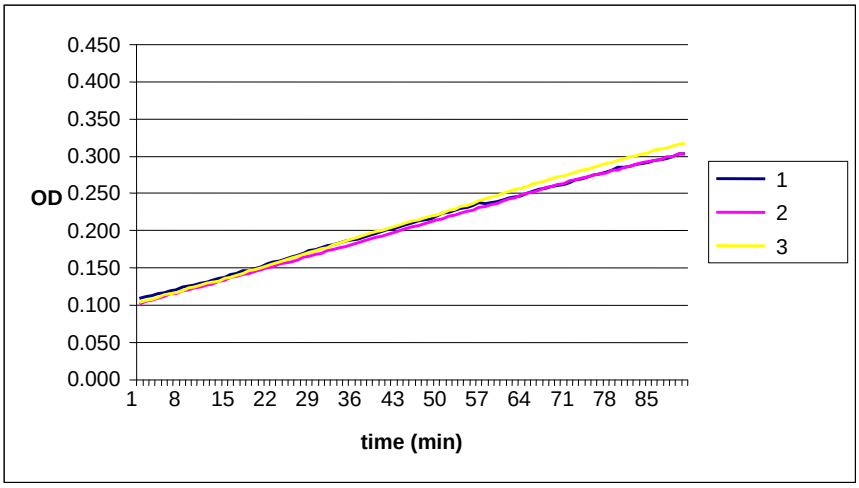


Figure 2-2 Example of LexA/$(LL)_4$ system.
Kinetic analysis of A1 transactivator line that was combined with R1 independent responder line of LexA/$(LL)_4$ system. This combination was analyzed three times that is shown as blue (1), red (2) and yellow line (3).
OD = 410 nm. 1, 2, 3 = independent extract measurement of A1R1 (see also Fig. 2-1).

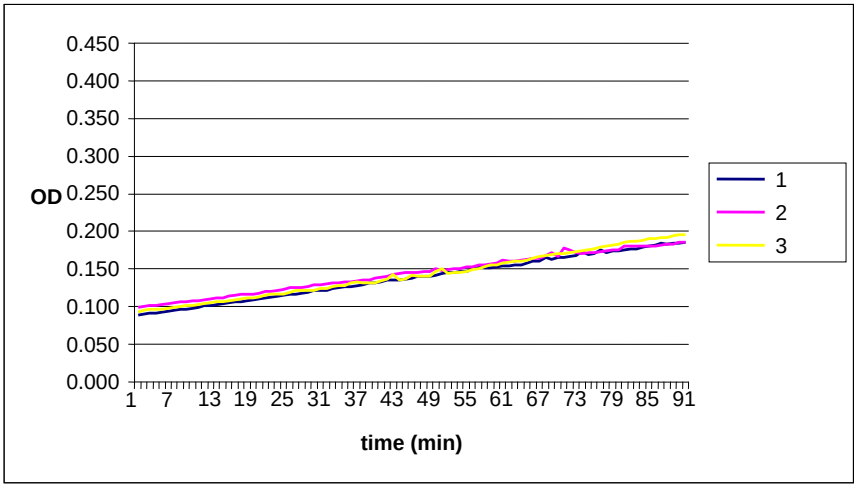


Figure 2-3 Example of LexA/*(LL)*$_4$ system.
Kinetic analysis of another transactivator line (A2) combined with the same responder line (R1) of the system as presented in Fig 2-2. This A2R1 combination showed very low β-gal activity in three independent extracts (1, 2, 3; see Fig. 2-1). OD = 410 nm.

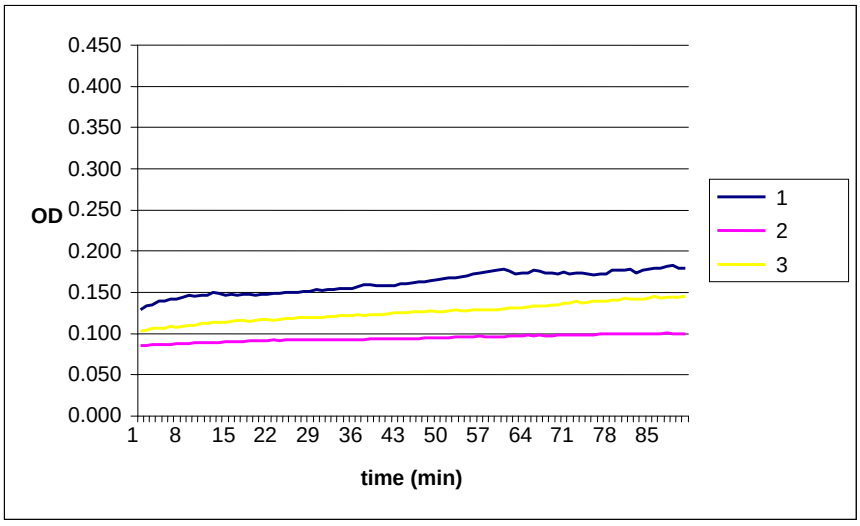


Figure 2-4 Example of *(LL)*$_4$ responder control.
Kinetic analysis of R1 responder line is indicated. Three independent extracts (1, 2, 3; see Fig. 2-1) were measured. OD range never exceeded 0.200 OD value. OD = 410 nm.

2.6.3 X-gal assay in DMSO (according to A. Schmitt, Karlsruhe, 2003)

This approach was established for *D. melanogaster*. In other insect species this should be tested prior to its usage there.

X-gal solution

10 mM Na-phosphate buffer pH 7.2
150 mM NaCl
1 mM $MgCl_2$
3 mM $K_4(Fe_2(CN)_6)$ x $3H_2O$
3 mM $K_3(Fe_3(CN)_6$
0.3% Triton X-100
10% X-Gal in DMSO (keep in dark at 4˚C)

Protocol

1) Mix 4 µl of X-Gal with 200 µl X-gal solution on the day when the assay is performed.
2) 50 µl/sample pipette into microplate.
3) Cut heads (if needed separate eyes from each other).
4) Heads place into wells with X-gal solution.
5) Cover the microplate with parafilm.
6) Let stand in dark overnight.
7) Check the colouration next day under binocular.

2.6.4 Immunoblotting

Head extracts

Six heads were cut with a cover slip and thoroughly homogenized in 15 µl of 1.5x SDS sample buffer in 500 µl tubes. After 5 min boiling and centrifugation at 14,000 rpm/ 5 min/ 4˚C, 10 µl supernatant was loaded per a lane.

SDS-PAGE (7.5%) and immunoblotting was carried out as described by Harlow and Lane (1988). As primary antibodies monoclonal mouse anti-β-galactosidase (1:1,000; Sigma), monoclonal mixture of anti-Tet repressor (1:500 or 1:1,000; MoBiTec) and as a secondary antibody, horse radish peroxidase-conjugated goat anti-mouse (1:2,000; Jackson Immunosearch) were used. As a loading control anti-α-tubulin (1:20,000; Amersham

Biosciences) was used. ECL membranes were provided by (Amersham Biosciences). Signals were detected on films (Kodak or Amersham Biosciences) by ECL™ detection kit (Amersham Biosciences).

2.7 MATERIAL AND METHODS FOR CHROMOSOMAL REARRANGEMENTS IN *D. MELANOGASTER*

To identify chromosomal rearrangements: (i) distinct fluorescent markers, EYFP, ECFP were placed under the universal eye-specific promoter, 3xP3 (Berghammer et al. 1999, Horn et al. 2000); (ii) fluorescent marker DsRed1 was placed under the control of constitutively active *polyubiquitin* promoter, PUb (Harrell and Handler 2001) and (iii) *FRT* sites were inserted into 5' UTRs, so that they separate the fluorescent marker from the promoter (Götschel 2003).

2.7.1 *Drosophila melanogaster* strains

2.7.1.1 Analyzed inversions and their chromosomal positions

X-linked inversion and other inversions on the third chromosome were analyzed (Tab. 2-4). *FRT* lines, which were used to create these inversions, are depicted in Fig. 2-5. Inverted chromosomal region between their *FRT* sites is indicated.

Table 2-4 Analyzed inversions on *X* and *III.* chromosomes

Inversion	Chromosome	Chromosomal insertion (cytobands)	Character of analyzed inversion
F1/F1	X	13D2/19E6	paracentric
F2/14	III. (3L)	69C7/69F5	paracentric
F2/26	III. (3L)	69C7/75E5	paracentric
13/1	III. (3R)	92A13/100B9	paracentric
14/1	III. (3L-3R)	69F5/100B9	pericentric
26/1	III. (3L-3R)	75E5/100B9	pericentric
F2/59	III. (3L)	69C7/70C8	paracentric
1/83	III. (3R)	100B9/100D1	paracentric

Inversions were the result of FLP/*FRT* mediated recombination between two *FRT* constructs. These were recombined onto one chromosome and the chromosomal region between their *FRT* sites was inverted. The *X*-linked inversion was created between the line F1 of *pBac[3xP3-FRT-ECFP]* construct and the line F1 of *pBac[PUb-FRT-DsRed*] construct (Götschel 2003). Other inversions were created between *FRT* sites of: (i) *pBac[PUb-FRT-DsRed]* construct (the line F2 and the line 1); (ii) *pBac[3xP3-FRT-ECFP]* construct (lines 13, 14 and the line 26) and (iii) *Herm[3xP3-FRT-EYFP]* construct (the line 59 and the line 83). These inversions were established by Götschel (unpublished data). Pericentric inversions include the centromere unlike paracentric ones.

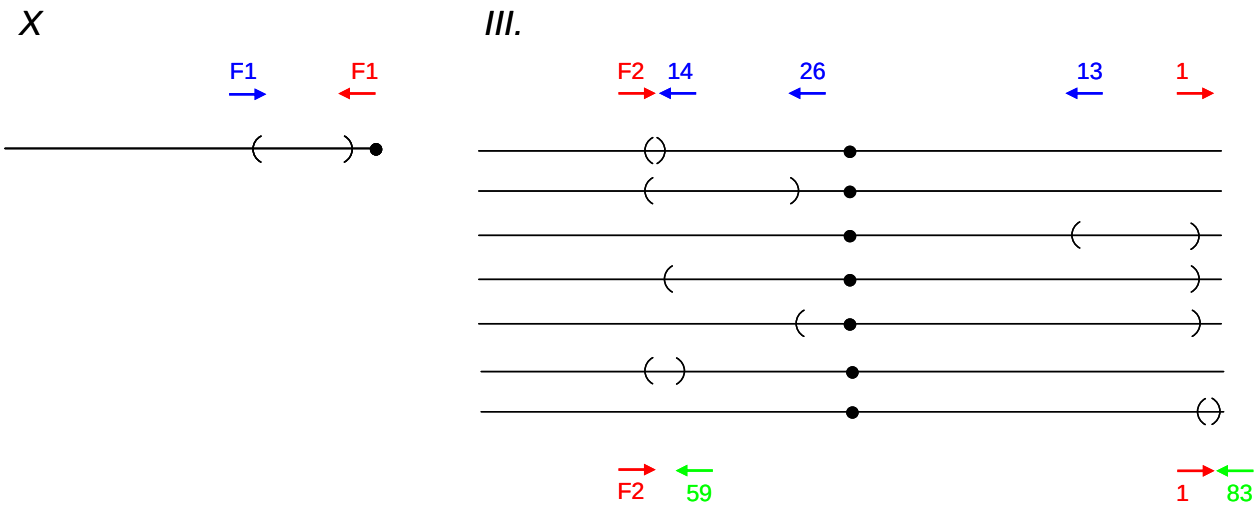


Figure 2-5 Chromosomal position of analyzed inversions on *X* and *III.* chromosome.
Two original *FRT* lines were recombined onto one chromosome and the chromosomal region between their *FRT* sites (arrows) was inverted by FLP/*FRT* mediated recombination. The length of such region is defined by parentheses on one chromosome. The orientation of *FRT* sites is indicated by the direction of an arrow. The colour of original *FRT* lines corresponds to the colour of their fluorescent markers, DsRed (red), ECFP (blue) or EYFP (green), respectively. *X* and *III.* chromosomes are drawn as lines and the centromere is signed as the back dot. See Tab. 2-4 for the description of *FRT* lines.

Table 2-5 Information about *FRT* lines

***FRT* line**	***FRT* construct**	**Genebank Accession Nr.**	**Insertion site of *FRT* construct**	**Locus_tag**	**Cytoband (3L)**	**Physical map position [bp]**
34	*pBac[3P3-FRT-ECFP]*	AE 003547	111,150	non-coding region of 104,318 bps	67E7	10,691,957
M2	*pBac[PUb-FRT-DsRed*]	AE 003547	273,365	non-coding region of 495 bps	67F1	10,854,172
24	*Her[3P3-FRT-EYFP]*	AE 003541	188,010	*CG10638* 187,318-190,231 2nd exon of isoform A 187,669-188,184	69C3	12,473,037
F2	*pBac[PUb-FRT-DsRed]*	AE 003541	243,778	*CG10632* 226,429-261,442 5th intron 230,297-260,459	69C7	12,528,805

The line 34 was inserted in non-coding region and proximally flanked by a gene, *ninA*. Insertion region of this gene is localized in 87497-90014 of AE 003547 in Genebank with annotation ID: CG6449. The line M2 was inserted in other non-coding region and distally flanked by a gene, *CG6409*. Both lines, 24 and F2, were inserted in genes. There already exist mutant alleles of these genes according to FlyBase. Interestingly, they were produced by *piggyBac* transposon mutagenesis. Mutants are referred to be viable and fertile.

2.7.1.2 Information about used *FRT* lines to create duplications/deletions

Two pairs of *FRT* lines on the third chromosome (3L) were used to establish defined deletions and duplications (Fig. 2-6). Detailed information about their insertion sites shows Tab. 2-5. *FRT* line combinations, M2/34 and 24/F2, are both within published deficiencies on web site page of DrosDel Isogenic Deficiency Kit (www.drosdel.org.uk, reviewed recently by Ryder et al. 2004). *Df*(3L)ED4457 represents a deleted region between cytobands 67E2-68A7 (ca 761 kb), which includes the region 67E7-F1 of M2/34 combination. *Df*(3L)ED4483 (ca 415 kb, 69A4-69D3) and *Df*(3L)ED4486 (ca 518 kb, 69C4-69F6), they both contain 69C3-C7 chromosomal region between 24/F2 combination. Therefore, no haplo-insufficient genes are expected for any of M2/34 and 24/F2 combinations.

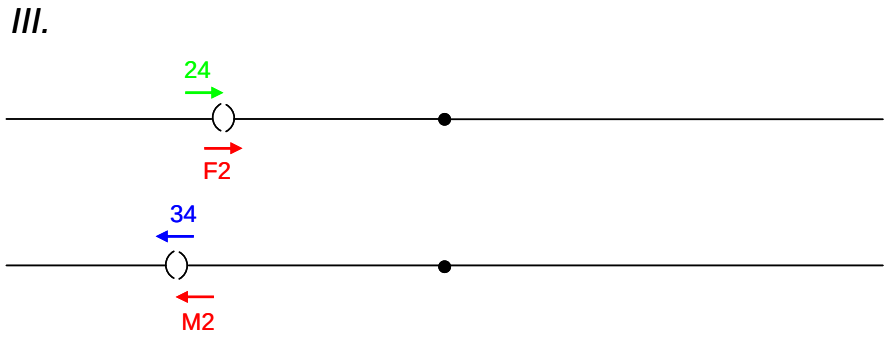


Figure 2-6 Chromosomal positions of used *FRT* lines to establish deletions and duplications.
Two pairs of *FRT* lines with the different insertion sites on the same chromosomal arm (3L) were used. Both *FRT* lines of one pair had their *FRT* sites (arrows) in the same orientation. Deletions of the chromosomal regions between *FRT* sites are shown. In the case of duplications, chromosomal arm will be prolonged about the length of these defined regions between *FRT* sites (not indicated). The line F2 and the line M2 = *pBac[PUb-FRT-DsRed]* construct; the line 24 = *Herm[3xP3-FRT-EYFP]* construct and the line 34 = *pBac[3xP3-FRT-ECFP]* construct. Depictions of constructs are given in Fig. 2-5. For information about *FRT* lines see Tab. 2-5.

2.7.2 Polytene chromosome squashes

Polytene chromosomes were prepared from four independent *In* (*X*)/FM7 balanced lines of *D. melanogaster*. Larvae were raised on the standard food with extra yeast at 18℃ till the third larval stage, which was used for the dissection of their salivary glands. The preparation of polytene chromosomes followed the protocol according to Wimmer (1995). Then, after dehydration in 90% ethanol (15 min), polytene chromosomes on slides were stained with 30 µl of Giemsa (Sigma) solution (1:50 ddH_2O) for 1 min under the cover slips. After this time, polytene chromosomes were destained for 2 min under running deionized water. After air drying, polytene chromosomes were embedded in 30 µl of Permout (Fisher Scientific) under the cover slip.

2.7.3 Crossing schemes and screening principles

2.7.3.1 Analysis of inversions to test their potential to act as partial balancers

Prior to create defined inversions by using FLP/*FRT* mediated recombination, *FRT* sites of two chosen *FRT* lines have to be recombined onto one chromosome (panel A, Fig. 2-7). Such situation will be called 'recombination' in the following. When these *FRT* sites are in opposite orientation to each other, FLP inverts the chromosomal region between them (panel B, Fig. 2-7). This situation will be called 'inversion' in the following. Thus, if the exact chromosomal positions of *FRT* sites are known, the inverted region is defined. When *FRT* sites are placed between a promoter and a fluorescent marker, the fluorescent marker possess an expression pattern before the inversion dependent on the promoter. After FLP/*FRT* recombination, fluorescent markers reciprocally exchange their promoters and this will be observed as the exchanged expression patterns of both fluorescent markers. This expression pattern is important to distinguish between the 'recombination' situation (panel A, Fig. 2-7) and its 'inversion' (panel B, Fig. 2-7).

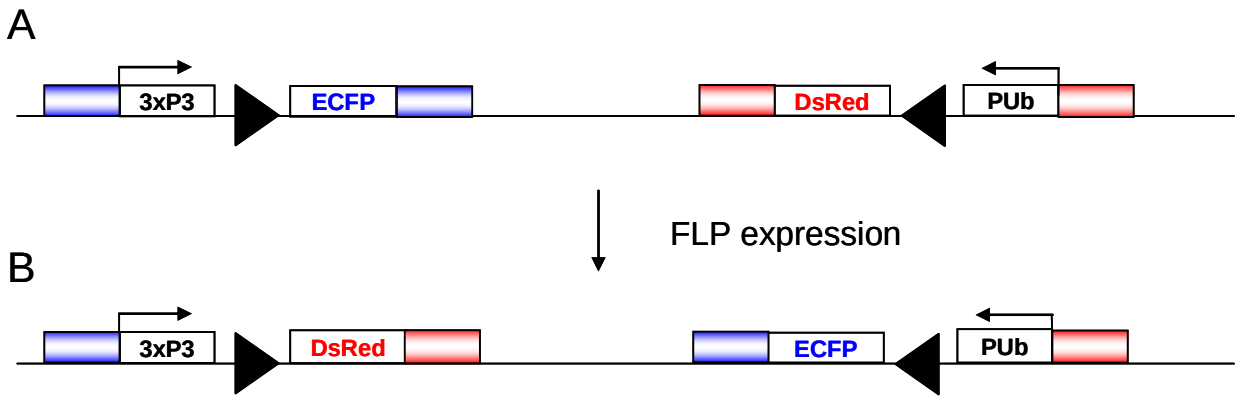
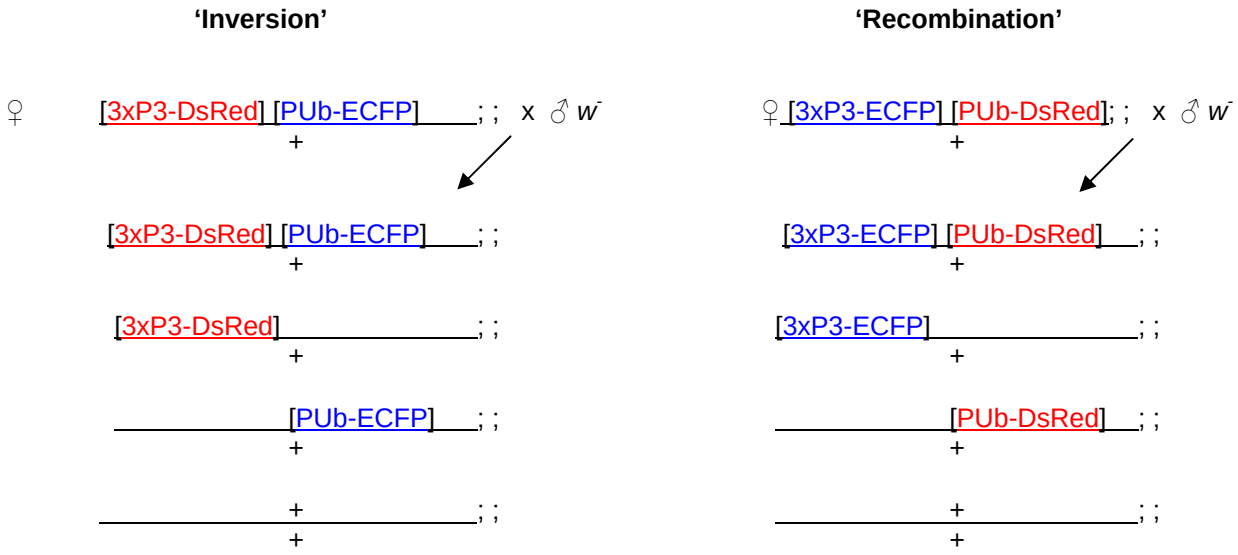


Figure 2-7 Inversions and their identification based on the exchange of promoters and fluorescent markers. Two distinct fluorescent markers (ECFP and DsRed) are placed under different promoters (3xP3 and *polyubiquitin*, PUb). Each fluorescent marker displays its expression pattern: ubiquitinously driven DsRed (PUb promoter) can be easily distinguished from ECFP expressed in the eyes (3xP3 promoter). If FLP/*FRT* mediated recombination invert chromosomal region between opposite oriented *FRT* sites (black arrow heads), fluorescent markers will reciprocally exchange their promoters and this will result in the exchange of their expression pattern. DsRed comes under the control of 3xP3 promoter while ECFP under the control of PUb.

'Inversion' situation was tested whether it can suppress meiotic recombination between its inverted chromosomal region and the non-inverted one on the homologous chromosome. As a control, 'recombination' situation was used. In this case, the meiotic recombination is expected. Thus, consequential segregation of fluorescent markers should occur. Because the meiotic recombination does not happen in *Drosophila* males, 'inversion' as well as 'recombination' situation was analyzed in heterozygous females according to the scheme:



3xP3-DsRed means *pBac[3xP3-FRT-DsRed]* construct and PUb-ECFP stands for *pBac[PUb-FRT-ECFP]* construct, these constructs have inverted *piggyBac* TIRs. 3xP3-ECFP and PUb-DsRed represent the original constructs, *pBac[3xP3-FRT-ECFP]* and *pBac[PUb-FRT-DsRed]*, respectively. This scheme shows the parental and the recombined genotypes. The progeny with one marker corresponds to a meiotic recombination while flies with both markers represent parental non-recombined situation. The same principle was for experiments on the 3rd chromosome. + = a wild-type chromosome.

2.7.3.2 Analyses of rearranged *piggyBac* terminal inverted repeats (TIRs)

When defined inversions between two *FRT* constructs are established, the inverted chromosomal region between their *FRT* sites results in the exchange of TIRs of these constructs (Fig. 2-8). 5' TIR of one *piggyBac* based construct and 3' TIR of other *piggyBac* based construct are placed between *FRT* sites (panel A, Fig. 2-8). When the chromosomal region is inverted by FLP/*FRT* recombination, rearranged *piggyBac* constructs are created (panel B, Fig. 2-8), 3'TIRs *piggyBac* and 5'TIRs *piggyBac*, respectively. They can be recognized according to switched expression pattern of used fluorescent markers (panel B, Fig. 2-8) and distinguished from the non-inverted situation (panel A, Fig. 2-8).

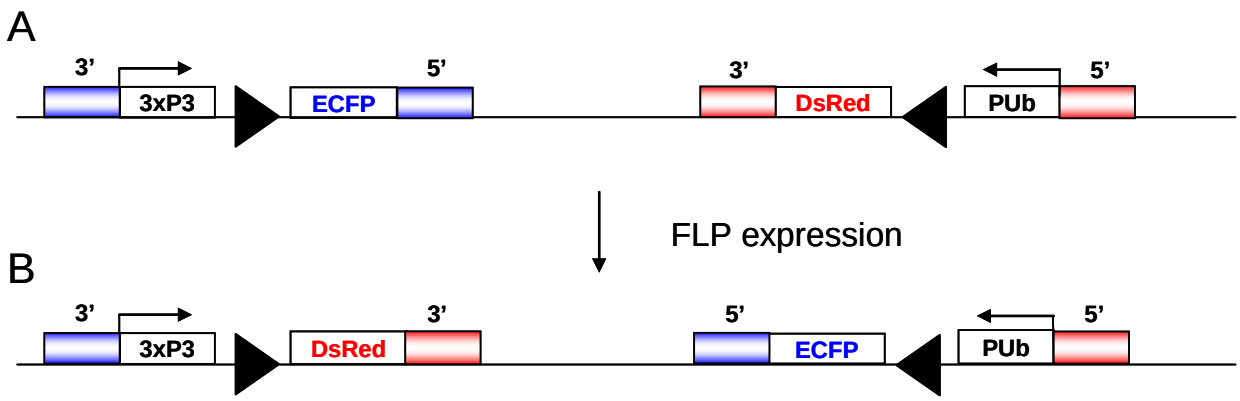


Figure 2-8 How 5' TIR and 3' TIR can be rearranged between two *piggyBac* constructs.
When the 5' terminal arm and 3' one are included between *FRT* sites of two *piggyBac* based constructs (A), as the consequent result of FLP/*FRT* mediated recombination will not only be exchanged fluorescent markers, but also 5' and 3' terminal arms (B). Thus, rearranged constructs will be created: *pBac3'-3xP3-FRT-DsRed-3'pBac* and *pBac5'-PUb-FRT-ECFP-5'pBac*. *FRT* sites are indicated as black arrows. Blue boxes show terminal arms of one *piggyBac* based construct and red boxes indicate terminal arms of the other one. 3xP3 and PUb = promoters; ECFP and DsRed = fluorescent markers.

Analysis of 3'TIRs piggyBac

To remobilize rearranged *pBac3'-3xP3-FRT-DsRed-3'pBac* construct, *piggyBac* transposase source was provided by line M6.II of jumpstarter stock, containing *Her[3xP3-ECFP;α-tub-piggyBacK10]* construct (Horn et al. 2003).

The crossing schema and principles of subsequent screen are shown in Fig. 2-9. To evaluate *piggyBac* transposase efficiency, the screening was based on *pBac[3xP3-FRT-DsRed]* construct, because this was distinguishable from *piggyBac* transposase source containing *Her[3xP3-ECFP;α-tub-piggyBacK10]* construct.

Analysis of 5'TIRs piggyBac

To remobilize rearranged *pBac5'-PUb-FRT-ECFP-5'pBac* construct, *piggyBac* transposase was provided by efficient line #1 containing *Mi[3xP3-DsRed; hsp70-piggyBac]* construct (Horn et al. 2003, Götschel 2003).

The crossing schemes and principles of subsequent screening are described in Fig. 2-10. In this case, the working efficiency of transposase source was based on the original *pBac[3xP3-FRT-ECFP]* construct, because this construct was easily distinguishable in 3xP3-DsRed background that was caused by *piggyBac* transposase source.

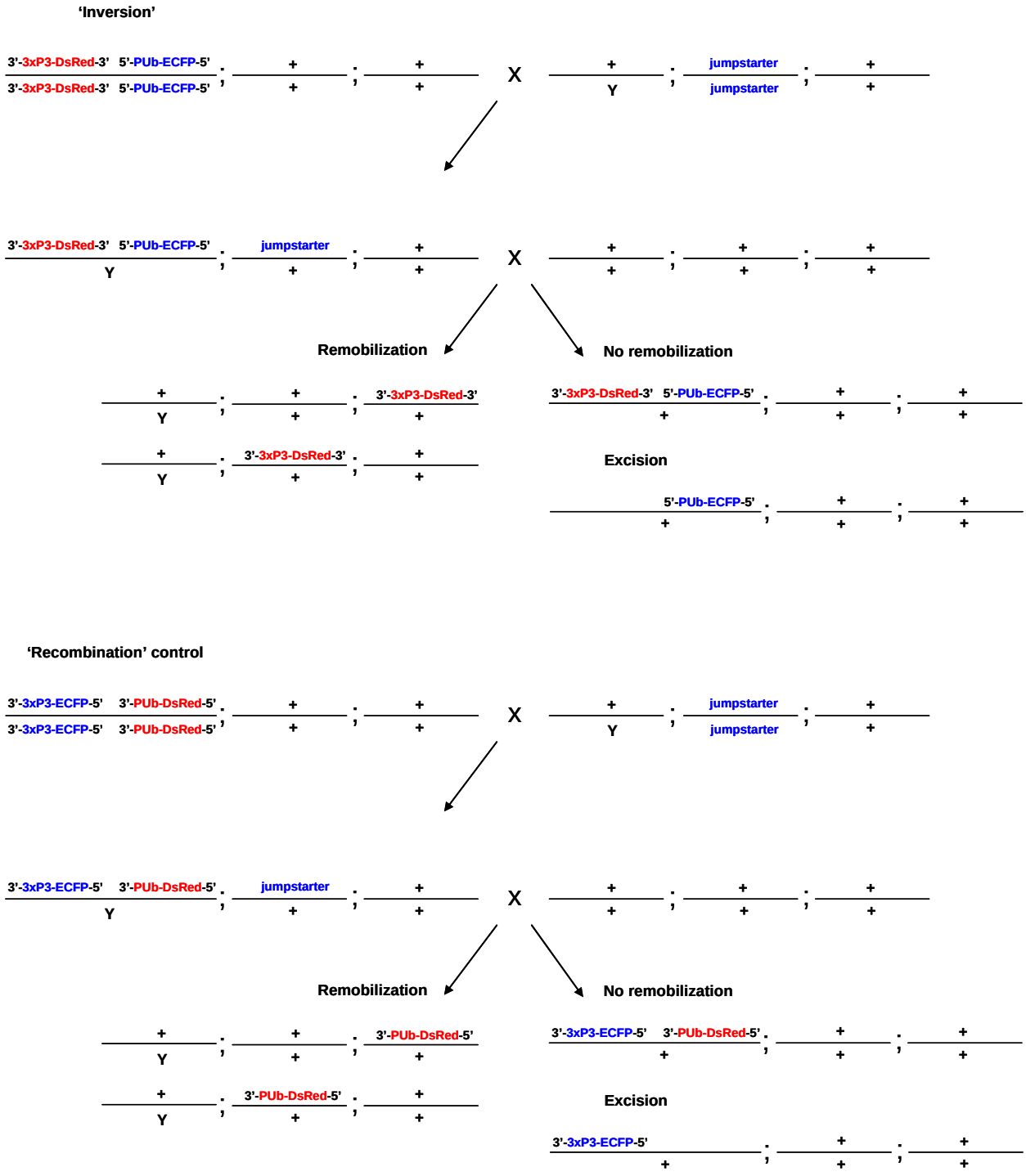


Figure 2-9 Analysis of rearranged *pBac3'-3xP3-FRT-DsRed-3'pBac* construct.
The crossing schemes were done for *X*-linked 'inversion' and 'recombination' situation. The latter served as a control. In the following, the construct names represent a line, which contained this construct. 3'-3xP3-DsRed-3'= rearranged *pBac3'-3xP3-FRT-DsRed-3'pBac* construct; 5'-PUb-ECFP-5' = rearranged *pBac5'-PUb-FRT-ECFP-5'pBac* construct; 3'-3xP3-ECFP-5' = the original *pBac[3xP3-FRT-ECFP]* construct and 3'-PUb-DsRed-5' = the original *pBac[PUb-FRT-DsRed]* construct. The jumpstarter line, which contained *Her[3xP3-ECFP;α-tub-piggyBacK10]* construct on the second chromosome, served as the *piggyBac* transposase source. The analysis was done in w^- background (not indicated) to be able to screen for the fluorescent markers. Resulting genotypes, which represent the remobilization, no remobilization and the excision, are indicated. + = wild-type chromosomes (w^- on *X* chromosome is not shown); Y = indicates male chromosome *Y*; x = a cross.

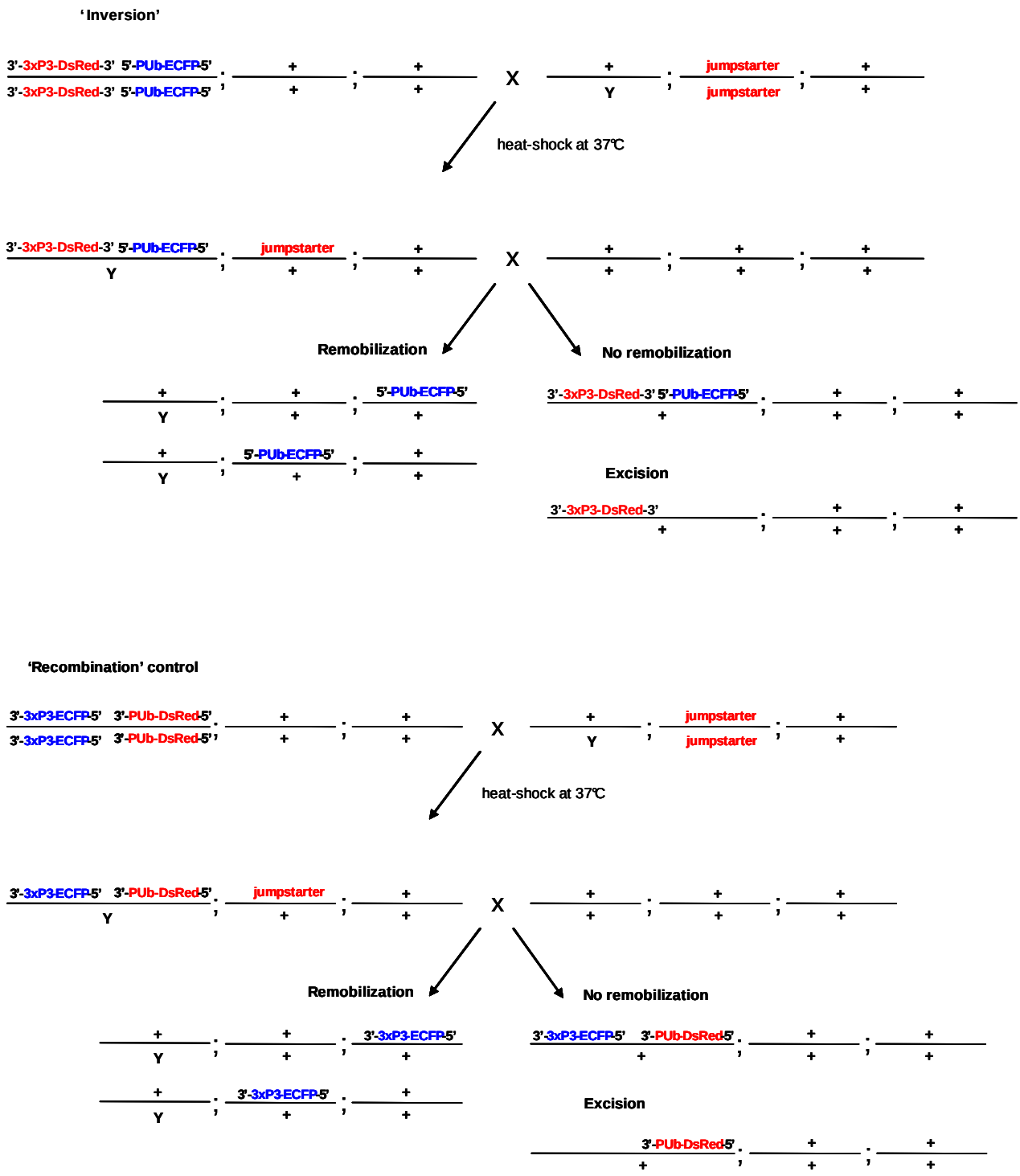


Figure 2-10 Analysis of rearranged *pBac5'-PUb-FRT-ECFP-5'pBac* construct.
The jumpstarter line contained *Mi[3xP3-DsRed; hsp70-piggyBac]* construct on the second chromosome. To activate *piggyBac* transposase expression, larvae were heat shocked at 37℃ incubator for 3 hours on three subsequent days. Descriptions and definitions of the crossing scheme were already presented in Fig. 2-9.

2.7.3.3 Analyses of rearranged TIRs between *piggyBac* and *Hermes*

By the establishment of inversions between two different constructs, *piggyBac* and *Hermes* based ones, their TIRs in the region between *FRT* sites of these constructs will be exchanged by FLP/*FRT* mediated recombination. When 3' TIR of each construct is included in this region (panel A, Fig. 2-11), rearranged *pBac5'-3'Her* and *Her5'-3'pBac* constructs will be created (panel B, Fig. 2-11). The crossing scheme and the principle of subsequent screening are described in Fig. 2-12.

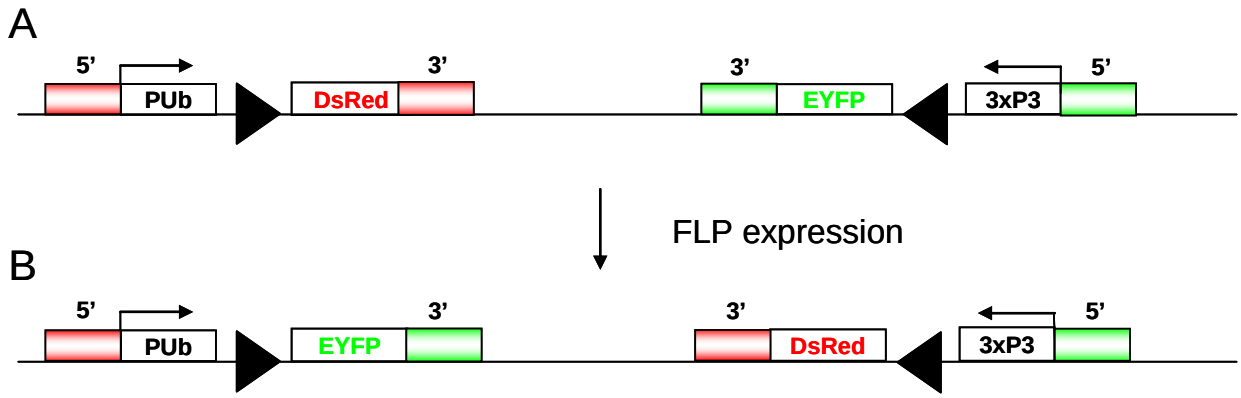
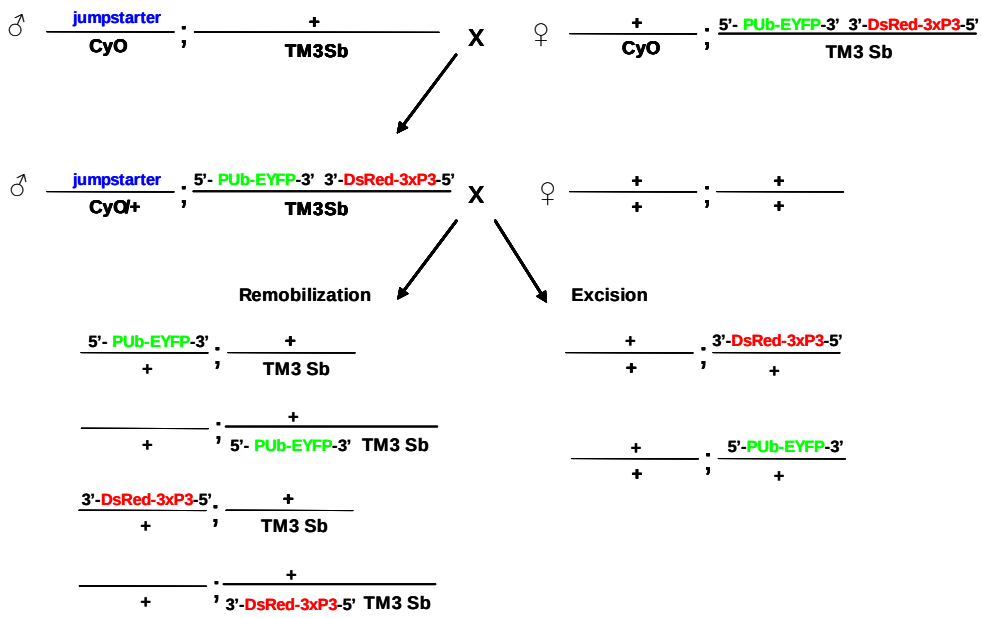


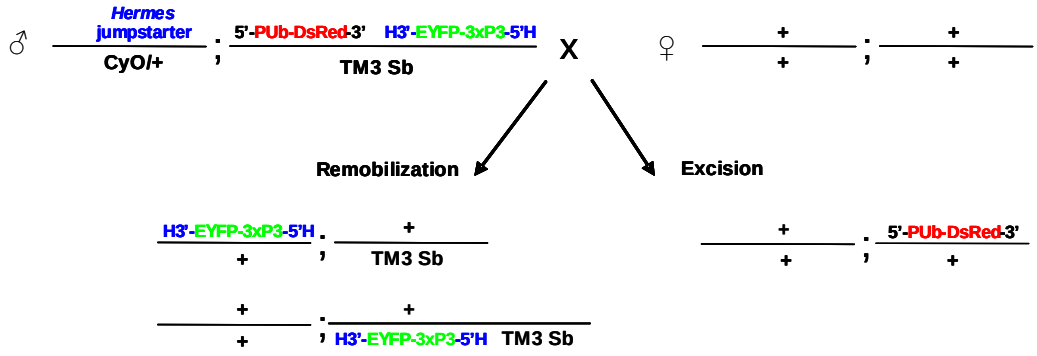
Figure 2-11 How *piggyBac* (red) and *Hermes* (green) constructs exchange their 3' TIRs.
In panel A, *piggyBac* construct (red boxes) is marked with PUb-DsRed while *Hermes* construct (green boxes) with 3xP3-EYFP. *FRT* sites are indicated as black arrow heads and are in opposite orientation to each other. If chromosomal region between these *FRT* sites is inverted by FLP/*FRT* mediated recombination, 3' terminal parts of *piggyBac* and *Hermes* construct will be exchanged (panel B). These chimeric constructs will be identified by the exchange of expression pattern of used fluorescent markers: DsRed (red), previously under the control of PUb promoter comes under 3xP3 control while EYFP (green), previously driven by 3xP3 promoter comes under the control of PUb promoter.

To provide *piggyBac* transposase, line M6.II containing *Her[3xP3-ECFP; α-tub-piggyBacK10]* construct was used (Horn et al. 2003). To provide *Hermes* transposase, line #5.II of *pBac[3xP3-ECFP;hsp70-Hermes]* construct was applied (Horn et al. 2003).

'Inversion'



'Recombination' control



'Recombination' control

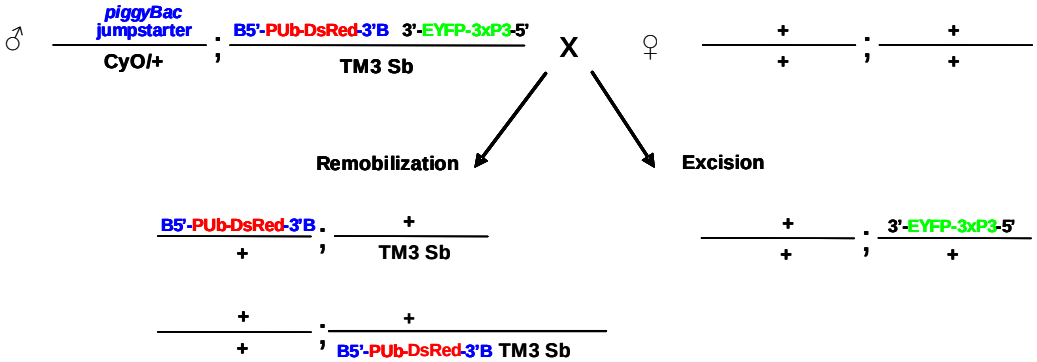


Figure 2-12 *pBac5'-3'Her* construct segregation from *pBac3'-5'Her* one.
If efficient *piggyBac* or *Hermes* transposase is provided, the segregation of rearranged constructs ('inversion') could theoretically happen. For simplicity, the remobilization of each construct on *X* chromosome in resultant female progeny is not indicated. 'Recombination' control is included. The jumpstarter on the second chromosome was 3xP3-ECFP marked and two types served as a source of the transposase, *Her[3xP3-ECFP; α-tub-piggyBacK10]* or *pBac[3xP3-ECFP; hsp70-Hermes]*. In latter case, larvae containing 'jumpstarter' and 'inversion' (or 'recombination') were heat shocked at 37℃ incubator for 3 h on thre e subsequent days. For simplicity, 'jumpstarter' and CyO are not shown in the resultant progeny. CyO and TM3 Sb indicate balancer chromosomes. + = the wild-type chromosome in w^- background (note that w^- mutation on *X* chromosome is not shown). In the following, construct names correspond to the line, which contained this construct: 5'-PUb-EYFP-3' = *pBac5'-PUb-FRT-EYFP-3'Her*; 3'-DsRed-3xP3-5' = *Her5'-3xP3-FRT-DsRed-3'pBac*; 5'-PUb-DsRed-3' = the original *pBac[PUb-FRT-DsRed]* construct; 3'-EYFP-3xP3-5' = the original *Her[3xP3-FRT-EYFP]* construct. H = *Hermes* TIRs; B = *piggyBac* TIRs. ♂ = male; ♀ = female.

2.7.3.4 Establishment of duplications and deletions

When two *FRT* sites are directly oriented and positioned in *trans*, i.e. on homologous chromosomes, induced FLP specifically recognizes these sites and mediates the site-specific recombination resulting in defined deletions and duplications (Senecoff et al. 1985, Golic and Lindquist 1989). The principle of the establishment of paracentric duplications and deletions by FLP/*FRT* recombination is depicted in Fig. 2-13.

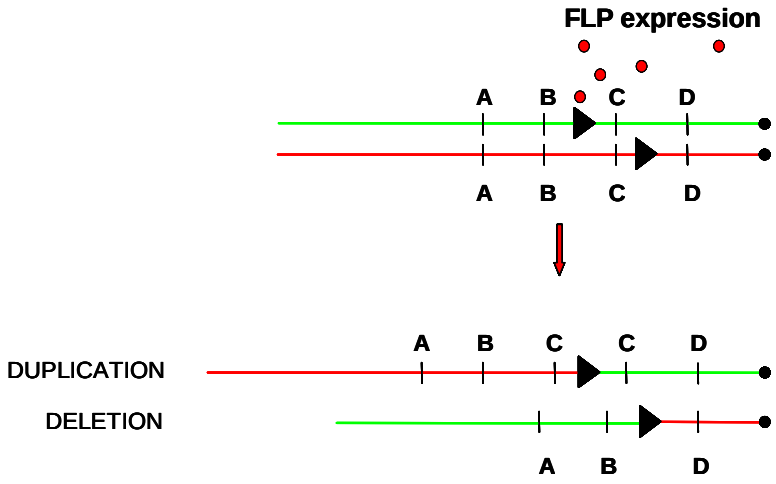


Figure 2-13 Paracentric duplications and deletions by site-specific FLP/*FRT* recombination.
FRT sites (arrows) possess the same orientation and are inserted on the same arm of homologous chromosomes (depicted as lines). One is inserted between region B and C whereas the other between C and D. Thus, if FLP source (red dots) is provided and specifically acts on *FRT* sites, it will cause both deletion of the C region or its duplication. Black dots right from D region correspond to the centromere. If both *FRT* sites had an opposite orientation, i.e. away from the centromere, it would result in the same duplicated and deleted chromosomal region by FLP/*FRT* recombination. Paracentric = the centromere is not included in the deleted or doubled region, therefore, no acentric or dicentric chromosomes are expected.

The attempt to establish duplications and deletions based on non-P-elements between homologous arms followed a scheme, where two fluorescent markers to distinguish between lines and two distinct promoters to identify the later chromosomal rearrangement were used (Fig. 2-14). As *FRT* sites were placed in between promoter and fluorescent marker, FLP-mediated recombination should cause an exchange between promoters resulting in a switch of fluorescent marker expression.

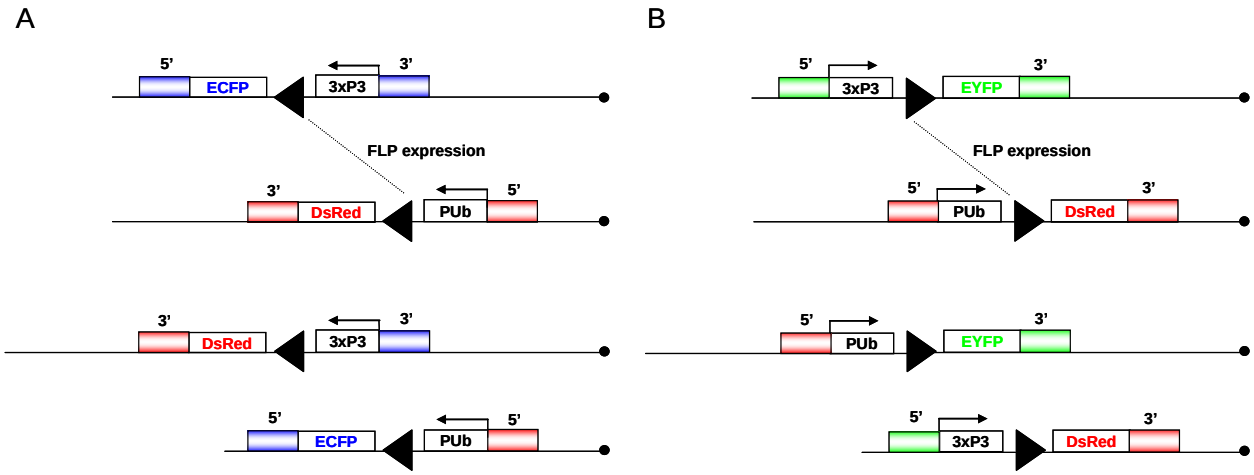


Figure 2-14 A switch of fluorescent markers to identify paracentric duplications and deletions.
In panel A, the line 34 (marked with 3xP3-ECFP) and the line M2 (marked with PUb-DsRed) lay on homologous chromosomal arms (depicted as full lines with dot as the centromere), but possess different insertion sites. When flipase source (FLP) is provided and the site-specific recombination on *FRT* sites (black arrows) occurs (dotted line), 3xP3 promoter will drive DsRed marker instead of previous ECFP and, on the other hand, PUb promoter will no longer drive DsRed, but ECFP. The switch of expression pattern of fluorescent markers allows identifying either the duplication (i.e. 3xP3-DsRed) or the deletion (i.e. PUb-ECFP). The resultant rearranged constructs, *pBac3'-3xP3-FRT-DsRed-3'pBac* and *pBac5'-Pub-FRT-ECFP-5'pBac*, contain only one type of the terminal arm. Note that both constructs are based on *piggyBac* transposable element (blue and red boxes). *FRT* sites are depicted as arrowheads corresponding to their orientation between promoter and marker. In panel B, the site-specific FLP/*FRT* recombination between the line 24 (marked with 3xP3-EYFP) and the line F2 (marked with PUb-DsRed) results in either the duplication (PUb-EYFP) or the deletion (3xP3-DsRed). In this case, *piggyBac* (red boxes) and *Hermes* (green boxes) based constructs were used. *FRT* sites are in the opposite orientation than in panel A. Note that the resultant rearranged constructs, *pBac5'-Pub-FRT-EYFP-3'Her* and *Her5'-3xP3-FRT-DsRed-3'pBac*, are created. 3xP3-ECFP = the original *pBac[3xP3-FRT-ECFP]*; Pub-DsRed = the original *pBac[Pub-FRT-DsRed]*; 3xP3-EYFP = *Her[3xP3-FRT-EYFP]*.

Crosses were done according to scheme presented in Fig. 2-15. *X*-linked *hsp70* promoter (DrosDel Kit) driven FLP recombinase was $y^{-}w^{-}$ marked. To activate *hsp70* promoter, the 2nd and 3rd instar larvae were heat shocked at 37℃ for 3 h on three subsequent days (4th - 6th day after crosses were set up).

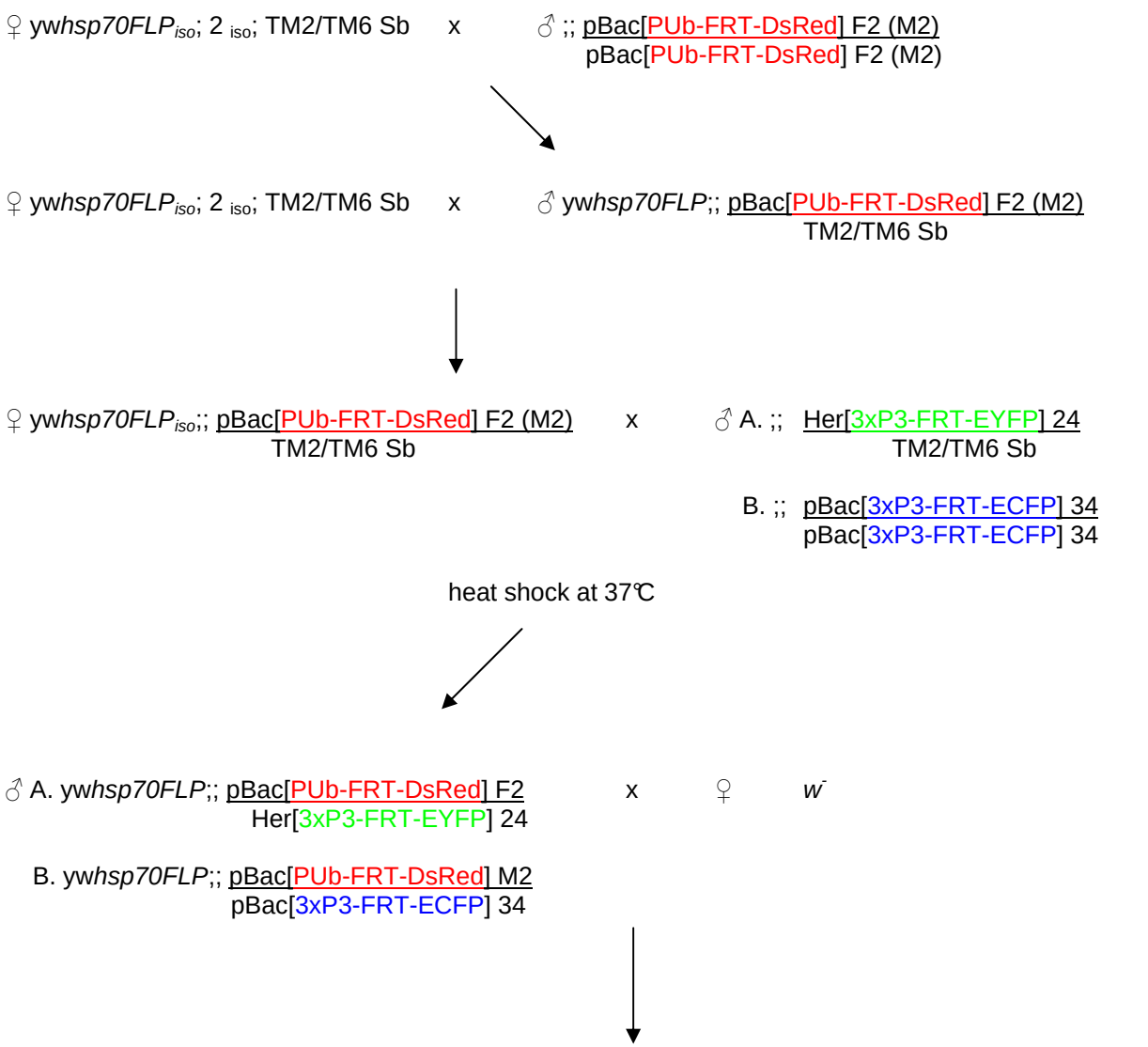


Figure 2-15 Crossing scheme with *hsp70* promoter driven FLP.
Both, line F2 and M2 in *piggyBac* (pBac) vector, were used for the first cross. In the following, the line F2 was crossed against *Hermes* (Her) based line 24 and the line M2 against *piggyBac* based line 34. In the case of 24/F2, TIRs of *piggyBac* and *Hermes* vectors were exchanged. Heat shock was applied at 37℃ incubator for 3h on three subsequent days. ♀ stands for virgins; ♂ = males; *iso* = isogenic line; TM2, TM6 Sb = balancer chromosomes; + = a wild-type chromosome.

3 RESULTS

3.1 TESTING OF A *DROSOPHILA* HEAT-SHOCK PROMOTER IN THE RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM*

To drive a correspondent transposase source of a non-autonomous mutator element to enable its remobilisation into another genomic site and trap an interesting enhancer or other regulatory sequences, a promoter particularly active in germ-line cells is necessary. In *Drosophila melanogaster* a temperature sensitive *hsp70* promoter is predominantly used. Its activation temperature in *Drosophila* is 37℃ (Ashburner 1989). The same temperature act ivates this promoter in stably transformed phytoseiid mites (Presnail and Hoy 1992). Recently, Uhlířová et al. (2000) have shown its functionality in somatic cells of the lepidopteran species *Bombyx mori*. However, a higher temperature of 42℃ is required in this moth. Whether this promoter functions also in *T. castaneum* and if so, where and under which conditions will be addressed in following result parts.

3.1.1 Germ-line transformation of *D. melanogaster*

To determine whether *Drosophila hsp70* promoter responds to the heat shock in a *piggyBac* based construct, six independent *D. melanogaster* lines carrying *pBac[3xP3-DsRed, hsp70-EGFP]* were tested (30A.X, M31.III, M19.III, M12.II, M18.III and M21.II). These lines displayed a variable DsRed1 intensity, pointing out a position effect as a result of different insertion sites in *Drosophila* genome. If the construct was correct, conditional activation of the *hps70* promoter should have resulted in EGFP expression. Heat shock was applied as described in Materials and methods (part 2.5). A weak EGFP expression was firstly observed in larvae after 10 h counted from the heat shock treatment. This confirmed the construct's correctness.

3.1.2 *Drosophila hsp70* promoter response to the heat shock in *D. melanogaster*

In this part, it was evaluated how various developmental stages and particular tissues respond to the heat shock in *D. melanogaster*.

3.1.2.1 Response during larval, pupal and adult developmental stages to the heat shock

All tested lines performed different induced EGFP expression, suggesting potential position effects. This confirms conditional activation of the *hsp70* promoter in *D. melanogaster*.

Intensity of EGFP expression did not correlate with intensity of 3xP3-DsRed marker, e.g. line 30A.X showed the strongest 3xP3-DsRed marker intensity, but its induced EGFP was the worst one when compared to other lines. Induced EGFP expression of each line differed during *Drosophila* development. The best induction of EGFP marker was observed in line M19.III for larvae while in line M21.II for pupae and adults (Fig. 3-1). Transgenic animals of these lines were heat-shocked as described in Material and methods (part 2.5). As controls were included non-heat-shocked transgenic stages and heat-shocked w^- mutants. In the heat-shocked w^- larva, a strong EGFP autofluorescence was observed in abdominal part under GFP2 longpass emission filter unlike under DsRed bandpass one. This was likely caused by ingested food, which is one of the general problems with the use of fluorescent markers (Horn et al. 2002). Optical lobes of CNS showed the strongest EGFP expression in all stages, to which contributed a transmission of 3xP3 driven DsRed fluorescent marker under GFP2 longpass emission filter. 3xP3-DsRed transformation marker expression was observed in CNS and additionally in anal plates of the larval stage and PNS of the pupal stage. This is consistent with similar observations for 3xP3-EGFP transformation marker (Horn et al. 2000).

3.1.2.2 Response to the heat shock in salivary glands of the 3rd larval instar

Salivary glands of heat-shocked transgenic, heat-shocked w^- mutant and non-heat-shocked 3rd larval instar were dissected (Fig. 3-2). Line M19.III was used. Strong induced EGFP expression was apparent in salivary glands of the heat-shocked transgenic larva (Fig. 3-2, A, Hs*) compared to the others (Fig. 3-2, A, non-Hs* and Hs-w-). No leakiness of *hsp70* promoter was observed as well as any autofluorescence.

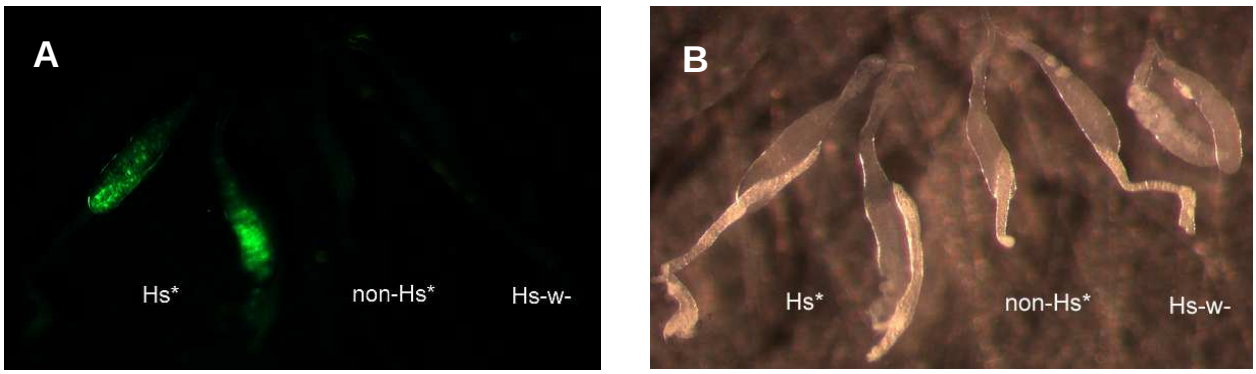


Figure 3-2 Salivary glands of the 3rd larval instar.
Strong induced EGFP expression was observed in salivary glands from heat-shocked transgenic larvae (Hs*). No constitutive EGFP expression was visible in those from non-heat-shocked (non-Hs*). No autofluorescence appeared in salivary glands of heat-shocked w^- larvae (Hs-w-). Line M19.III is shown.

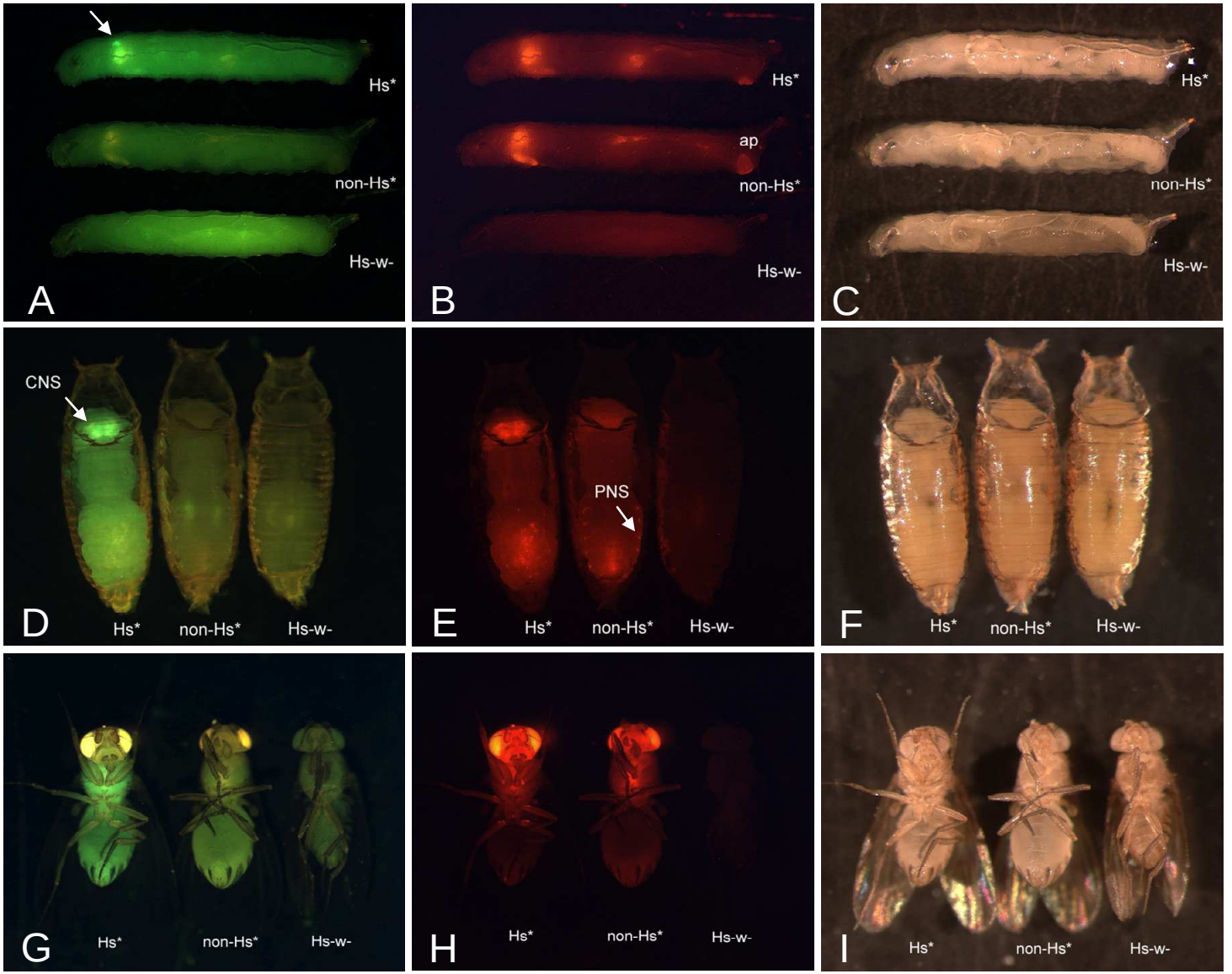


Figure 3-1 Heat-shock response in larval, pupal and adult stages of *Drosophila melanogaster*.
Heat-shocked transgenic (Hs*) and non-heat-shocked transgenic (non-Hs*) stages were compared with heat-shocked white mutant (Hs-w-). A, D, G images were taken under GFP2 filter. B, E, H correspond to DsRed filter while C, F, I to cold light. Larval stage of line M19.III: A, B, C. Pupal stage of line 21.II: D, E, F. Adult flies of line 21.II: G, H, I. In image A, bright heat-shock induced EGFP expression is visible in larval brain (arrow); in image B, anal plates are indicated as 'ap' in Hs* and non-Hs* larvae. In image D, the arrow shows central nervous system (CNS) in Hs* pupa; in image E, PNS (arrow) represents peripheral nervous system in non-Hs* pupa.

3.1.2.3 Response in *Drosophila* reproductive organs to the heat shock

hsp70 promoter is also used in *Drosophila* genetics to drive conditional expression of any gene of one's interest in germline during oogenesis. The evaluation of which parts of *Drosophila* reproductive organs do respond to the heat shock based on *hsp70*-EGFP expression is addressed in the following. Adult flies, which were used, belonged to line M21.II.

Female reproductive organs

Dissected *Drosophila* ovaries from heat-shocked transgenic and *w*⁻ flies were compared with those from non-heat-shocked transgenic flies (Fig. 3-3). The strong induced EGFP expression was visible in S13/14-like oocytes from *Drosophila* ovaries (Fig. 3-3, A, Hs*). No constitutive EGFP expression was apparent. Induced EGFP expression is observable in follicle cell layer on the very apical tip of S13/14 oocytes (Fig. 3-3, A, Hs*, arrow).

Wang and Lindquist (1998) found that nuclear transport of the heat-shock factors (HSF) controls the inducibility of Hsp70. They showed that HSF move out of nuclei to the cytoplasm in S10-S11 oocytes. Therefore, oocytes should not be heat shock inducible at this stage. First HSF relocalization from cytoplasm to nuclei appears again in pole cells of embryo at cycle 12. Based on that, thus, the visible expression of EGFP reporter in S13/14 oocytes is a result of the heat-shock response in oocytes of earlier stages than S10/11. It is reasonable, because the dissection of *Drosophila* ovaries was carried out 24 h after the heat shock treatment. Oocytes at stage S13/S14 displaying EGFP were likely heat shocked around S8 stage.

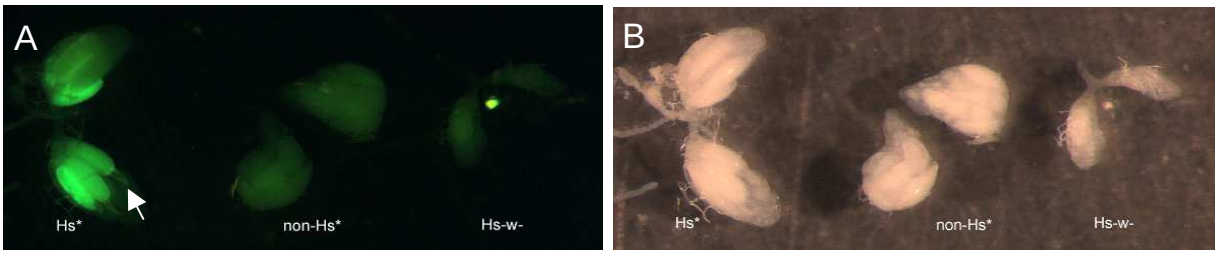


Figure 3-3 *Drosophila* adult ovaries.
Heat-shocked (Hs*) and non-heat-shocked (non-Hs*) transgenic adult flies of line M21.II were dissected for ovaries and compared to heat-shocked ones of *w*- females (Hs-w-). In picture A, visible follicle cell layer is indicated by arrow. A, GFP2 filter; B, cold light. For detail description see text.

Male reproductive organs

Adult testes were dissected from heat-shocked and non-heat-shocked transgenic flies as well as heat-shocked *w*$^{-}$ flies and compared (Fig. 3-4). In *Drosophila*, testes have a coiled tube appearance. There is also one pair of accessory glands called paragonia (Fig. 3-4, A, Hs*, p). Testes of heat-shocked transgenic adults showed similar EGFP expression as those of non-heat-shocked transgenic adults (Fig. 3-4, A, Hs* vs. non-Hs). Evidently, *hsp70* promoter was leaky. This was observed in other tested lines as well. EGFP marker varied in its intensity in these lines (data not shown).

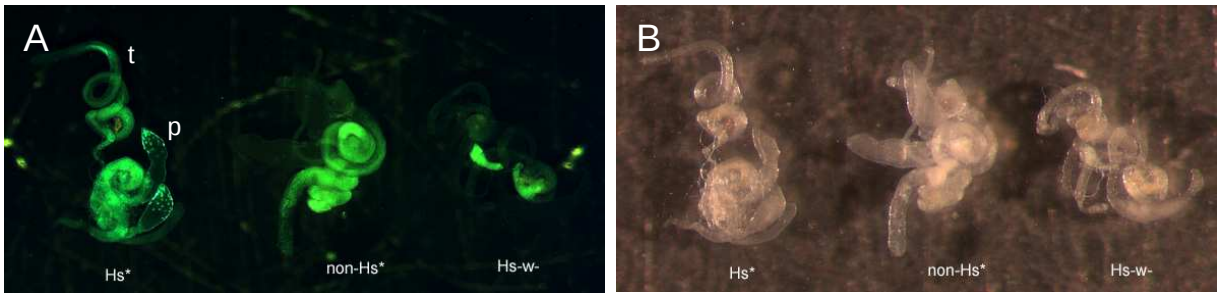


Figure 3-4 *Drosophila* testes and their accessory glands.
Strong induced EGFP expression as well as constitutive one was observed in adult testes of line M21.II (t). Heat-shock induced EGFP expression was visible in paragonia (p) representing *Drosophila* accessory glands. In testes of heat-shocked *w*$^{-}$ adults, autofluorescence was displayed in a part of coiled testis tubes. A, GFP2 filter; B, cold light.

Strong induced EGFP expression was visible in *hsp70* line M21.II in paragonia, the accessory glands of *Drosophila* testes, suggesting potential position effect. Interestingly, Hrdlicka et al. (2002) presented Gal4 line, which displayed the same pattern when this was crossed against *UAS*-mCD8-GFP responder (Lee and Luo 1999). They concluded that the pattern corresponds to the strong reporter expression in the secondary secretory cells of the accessory glands while to the weak expression in main cells of these glands. Gal4 line as well as *hsp70* line (this thesis) was inserted on the 2nd chromosome.

3.1.3 Germ-line transformation of *T. castaneum*

To determine whether *Drosophila hsp70* promoter functions in *T. castaneum* and which temperature is necessary for *hsp70* activation, firstly, transgenic lines containing *pBac[3xP3-DsRed;hsp70-EGFP]* construct were generated (Tab. 3-1).

Table 3-1 Transformation efficiency of *pBac[3xP3-DsRed;hsp70-EGFP]* construct

Construct name	No. of injected eggs	No. of hatched larvae	No. of eclosed pupae	No. of crosses set up	No. of sterile crosses	No. of transgenic lines	Transg. line name	Transg. line available
3xP3-DsRed;*hsp70*-EGFP	1252	128 (10.2%)	76 (59.4%)	72 (56.3%)	4 (5.6%)	4 (5.9%)	A	yes
							B	yes
							C	no
							D	yes
							E	yes

Transformation efficiency (%) = No. of transgenic lines/No. of fertile crosses (4/68). The percentage of sterile crosses = No. of sterile crosses/No. of crosses set up. The percentage of hatched larvae = No. of hatched larvae/No. of injected eggs. All other percentages = No. of pupae or crosses/ No. of hatched larvae. No. = number. Transg. = transgenic line; C did not survive.

3.1.4 *Drosophila hsp70* promoter response to the heat shock in *T. castaneum*

To screen for *hsp70*-EGFP expression, pupae of all available independent lines were tested. This developmental stage was chosen, because there is no pigmentation yet and therefore fluorescent marker expression is easy-to-detect unlike in beetles, where the body is covered by non-transparent dark cuticle. Moreover, pupae are the first stage, which can be easily sorted for gender (Sokoloff, 1972). And importantly, their reproductive organs show already similar anatomy to the ones in adult beetles.

In regard to beetle natural and laboratory conditions, a dry heat-shock was applied (i.e. pupae in vials were exposed to higher temperatures in incubator) to avoid possible pupae suffocation that may be caused by condensation water during a heat-shock in a water-bath. Several temperatures from 42℃ to 50℃ were tested for different time periods of 1 to 3 hours. Heat-shocked pupae were checked for EGFP production within 24 h under a fluorescent stereomicroscope.

To activate *hsp70* promoter from *D. melanogaster* in *Tribolium castaneum*, an exposure to temperature of 47℃ for 1 h in the incubator was found to be sufficient with a subsequent recovery of heat-shock treated pupae in 25℃ incubator. This was evaluated as an optimal condition, which means: (i) exposed pupae survived the heat-shock treatment, (ii) were able to reach adulthood afterwards and (iii) a visible EGFP expression was observed by 24 h after heat-shock treatment. A final comparison of all 4 independent lines tested is shown in Fig. 3-5.

In this figure, transgenic pupae (Hs*) as well as pupae of *vermilion*^{white} mutant (Hs-vw) were heat shocked according to found conditions above. To control a constitutive expression of this construct, transgenic non-heat-shocked pupae (non-Hs*) were included. The *hsp70*-EGFP expression was found to be different relatively to each line. This reflects potentially different enhancer traps or position effects related to the construct insertion site in the genome. The best activation of the *hsp70* promoter showed line A. In this case, the strongest induced EGFP expression (Hs*) and almost no constitutive expression (non-Hs*) was observed. On the other hand, the strongest leakiness of *hsp70* promoter was observed in line D (non-Hs*), which was comparable to the intensity of induced EGFP expression (Hs*). This line performed the lowest inducibility of the *hsp70* promoter.

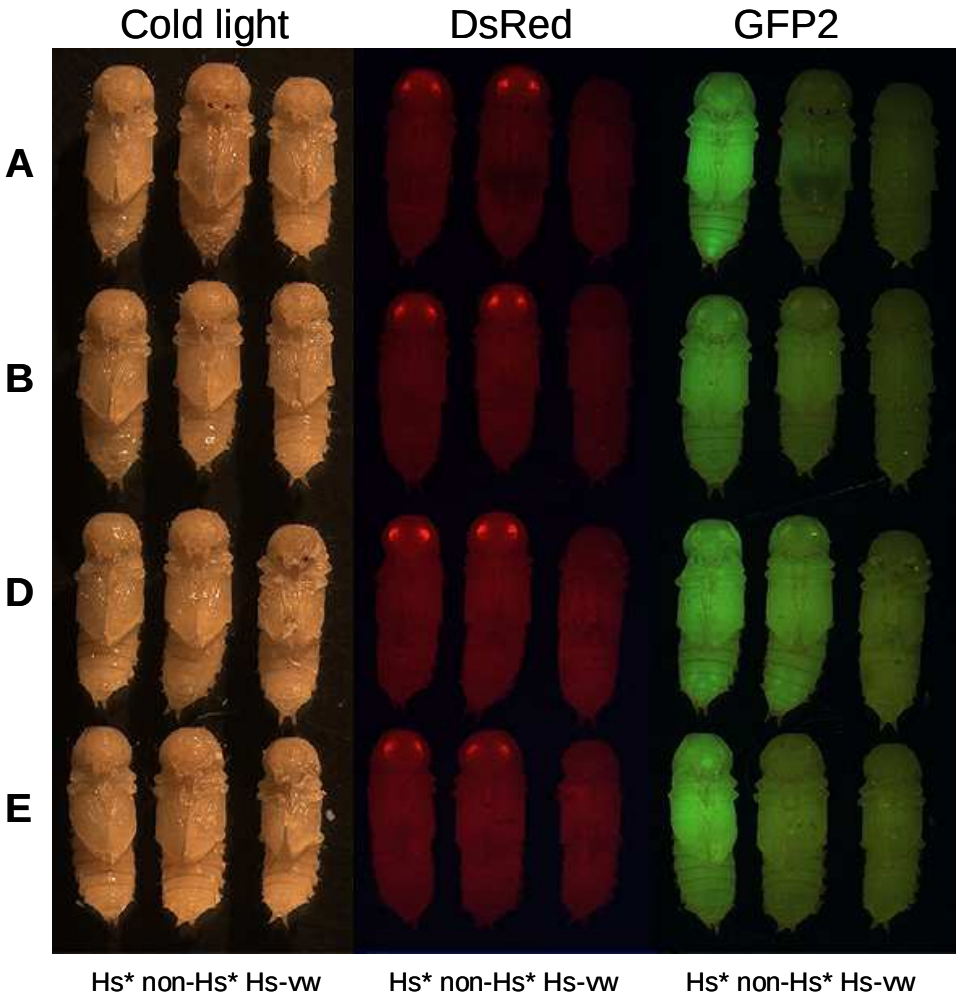


Figure 3-5 A comparison of four independent *Tribolium* lines after the heat-shock treatment.
All lines contain the *pBac[3xP3-DsRed;hsp70-EGFP]* construct and were compared to *vermilion*^{white} mutants (vw). Heat-shock was applied for 1 h at 47℃ in incubator. Hs*, heat-shocked transgenic line; non-Hs*, non-heat-shocked transgenic line; Hs-vw, *vermilion*^{white} heat-shocked control line. On left, cold light image; in the middle, DsRed filter image and on right GFP2 filter image is presented.

Furthermore, to address whether other developmental stages also respond to the heat-shock treatment, larvae and adult beetles were treated under the same heat-shock conditions as pupae. A comparison of the EGFP marker expression of larval, pupal and adult stages is shown in Fig. 3-6. In the larval stage, EGFP expression was visible in the whole thoracic and abdominal parts and in the eyes. In the beetle stage, visible EGFP expression appeared in inner tissues. Prior to a fluorescent marker check, the elytra of adult beetles were removed. Finally, a leakiness of this promoter was detected in this stage.

3.1.4.1 *Drosophila hsp70* response to the heat shock in *Tribolium* reproductive organs

As shown above, *hsp70* promoter from *Drosophila melanogaster* is functional in *Tribolium castaneum*. However, to drive *hsp70*-transposase source corresponding to a non-autonomous 'mutator' element on condition that new genomic insertions of the 'mutator' element are heritable, *hsp70* promoter induction has to work in germ-line cells. To evaluate which parts of male and female reproductive organs possess such response, dissections of heat-shocked (Hs*), non-heat-shocked (non-Hs*) transgenic pupae and heat-shocked *vermilion*^{white} (Hs-vw) pupae followed. The *hsp70* driven EGFP expression was checked after 24 h counted from the heat-shock treatment.

Female reproductive organs

As shown in Fig. 3-7, female reproductive organs of transgenic line A specifically responded to the heat shock. Almost ubiquitous strong inducible EGFP expression occurred in bursa copulatrix, spermatheca, spermathecal gland and ovaries while, it was slightly decreased in oviducts. Importantly, EGFP expression was also induced in each ovariol. *Tribolium castaneum* ovary consists of six ovariols as described in Sokoloff (1972). According to investigations of J. Büning lab, this species has telotrophic ovaries with two parts, tropharium and vitellarium, respectively. Each ovary contains approximately 30-40 oocytes placed in the posterior part of the tropharium. During oogenesis these oocytes grow and are accompanied by nurse cells, which migrate towards the anterior part of the oocyte. Fig. 3-8 shows ovaries of different pupal stages in detail. Induced EGFP marker expression was spatially enlarged in the heat-shocked pupae of younger stage (Fig. 3-8, A) while in older oocyte stage disappeared in apical part (Fig. 3-8, B). This likely corresponds to the heat-shock response of the nurse cells, their migration within 24 h after the heat shock as well as their degradation in older oocyte stages (J. Büning, personal comm.).

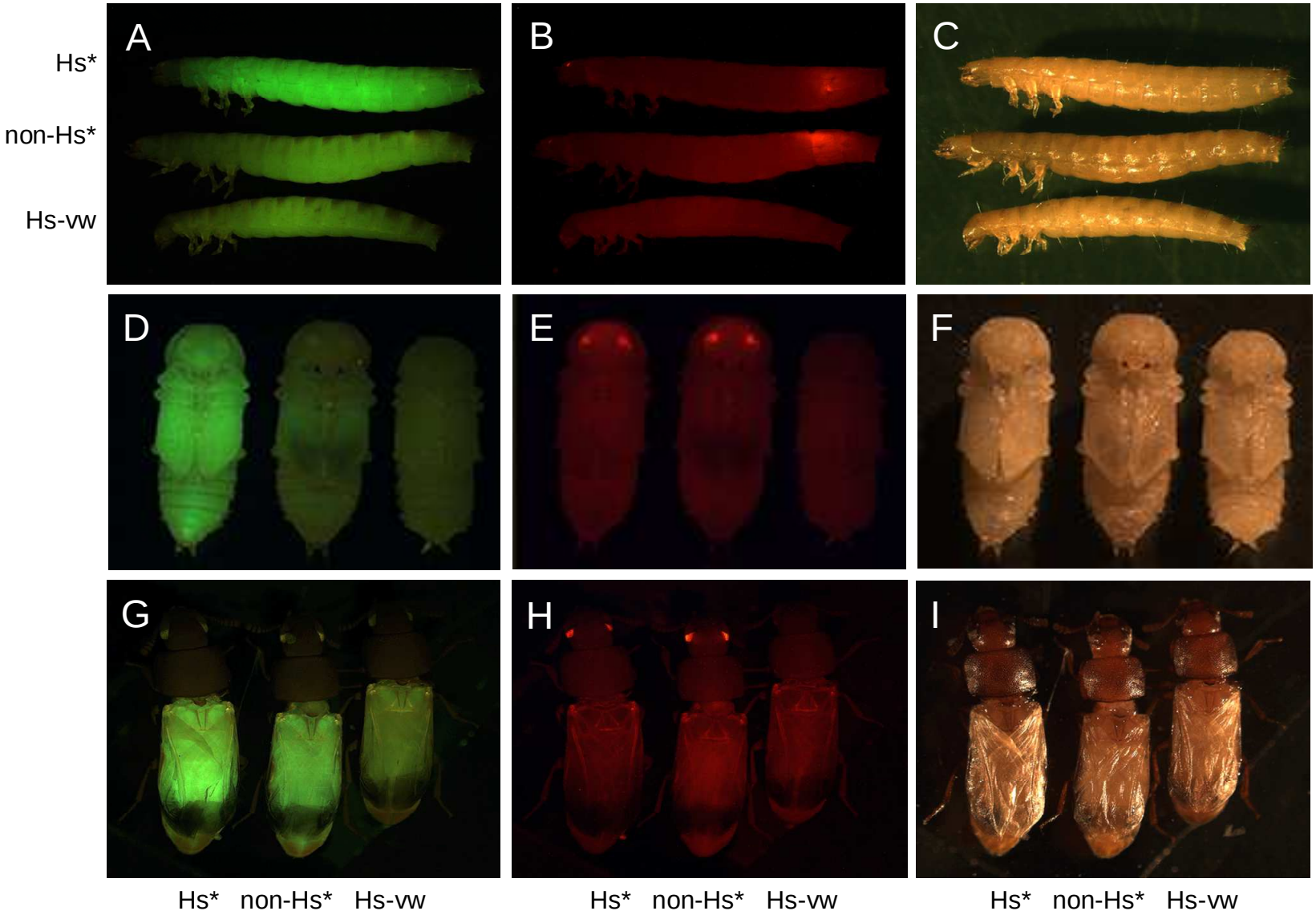


Figure 3-6 Heat-shock response in larval, pupal and adult stages of *Tribolium castaneum*.
All heat-shocked transgenic stages (Hs*) presented do display specific *hsp70*-EGFP expression as a response to the heat-shock. Non-heat-shocked transgenic larva (non-Hs* in A) and heat-shocked *vermilion*^{white} larva (Hs-vw) showed autofluorescence. Non-heat-shocked beetle (non-Hs* in G) showed that *Drosophila hsp70* is leaky in *T. castaneum*. Larvae, line B; pupae, line A; beetles, line D. To see EGFP marker in beetles, elytra were removed. Strong 3xP3-DsRed expression is visible in anal part of larval stage in addition to fluorescence in stemmata (Hs*, non-Hs* in B). GFP2 filter: A, D, G; DsRed filter: B, E, F; cold light: C, F, I. In the bottom: a description for pupae and beetles. In the top left corner: larval description.

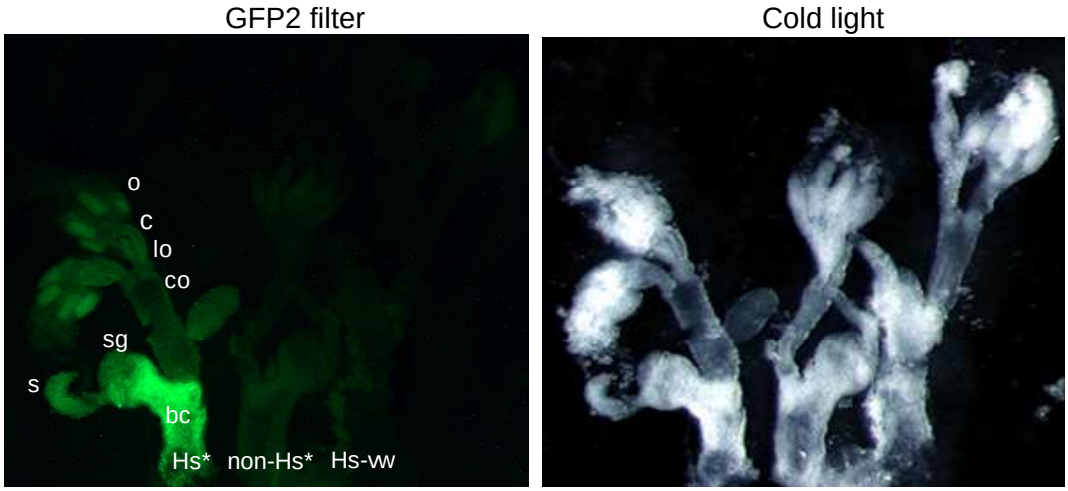


Figure 3-7 Female reproductive organs in *Tribolium castaneum*.
Ovaries of heat-shocked transgenic pupae (Hs*) showed visible *hsp70*-EGFP marker intensity compared to non-heat-shocked transgenic (non-Hs*) and heat-shocked *vermilion*^{white} ovaries (Hs-vw). The highest intensity was visible in bursa copulatrix (bc), spermatheca (s) and spermathecal gland (sg). Importantly, germ-cells in ovaries (o) likely responded to the heat-shock as well. Calyx (c), lateral oviduct (lo) and common oviduct (co) reacted with less fluorescent marker intensity to the heat-shock treatment.

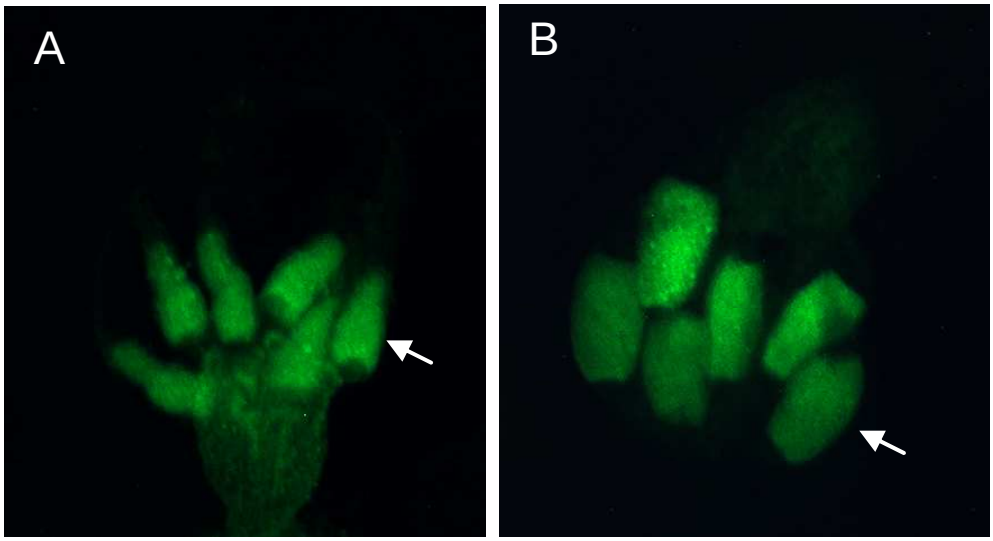


Figure 3-8 Heat-shock response in *Tribolium* ovaries.
In picture A and B is visible inducible EGFP expression (the heat-shock response) in ovaries of different ages. Picture A represents younger stage with potential nurse cells that responded to the heat shock. Picture B corresponds to an older stage of *Tribolium* oocytes (arrow) than in the picture A. Six ovariols of line A are shown.

Male reproductive organs

Testes of *Tribolium* pupae have already developed two pairs of accessory glands as well as two testes consisting each of six follicles (Fig. 3-9). Typical grape-shape testis is created by a connection and an extension of follicles to vas deferens. To check EGFP expression in male reproductive organs, transgenic line D was dissected. A strong EGFP expression was observed in accessory glands, ejaculatory duct, seminal vesicles and vas deferens (Fig. 3-9, Hs*). However, not all EGFP expression was heat-shock induced, because a leakiness of *hsp70* promoter was apparent especially in vas deferens and accessory glands (Fig. 3-9, non-Hs*). Thus, EGFP basal expression contributed to the observed intensity of EGFP expression in heat-shocked male reproductive organs. It is not as surprising, because line D performed the lowest inducibility and the highest leakiness of *hsp70* promoter. Nevertheless, a strong heat-shock specific response was found in apically placed cells of testicular follicles. These were probably supporting hub cells or a mitotic proliferation zone, giving rise to cyst cells enclosing later spermatocytes (for detail see Fig. 3-10). Germ-line cells should be placed in the middle area of testicular follicles (J. Büning, personal comm.), where no induced EGFP expression was observed. However, spermatozoa could have migrated within 24 h after the heat shock treatment to the vas deferens. Almeida and Cruz-Landim (2000) showed by using SEM that spermatozoa of *Tenebrionidae* (Coleoptera) and spermatophores with spermatozoa (a primitive method of the insemination in *Tribolium castaneum*) were found in place of vas deferens. Nonetheless, whether germ-line cells specifically reacted to the heat-shock is questionable, which is emphasized by the fact that line D performed the highest basal EGFP expression and the least inducibility. Detailed experiments with line A will be needed.

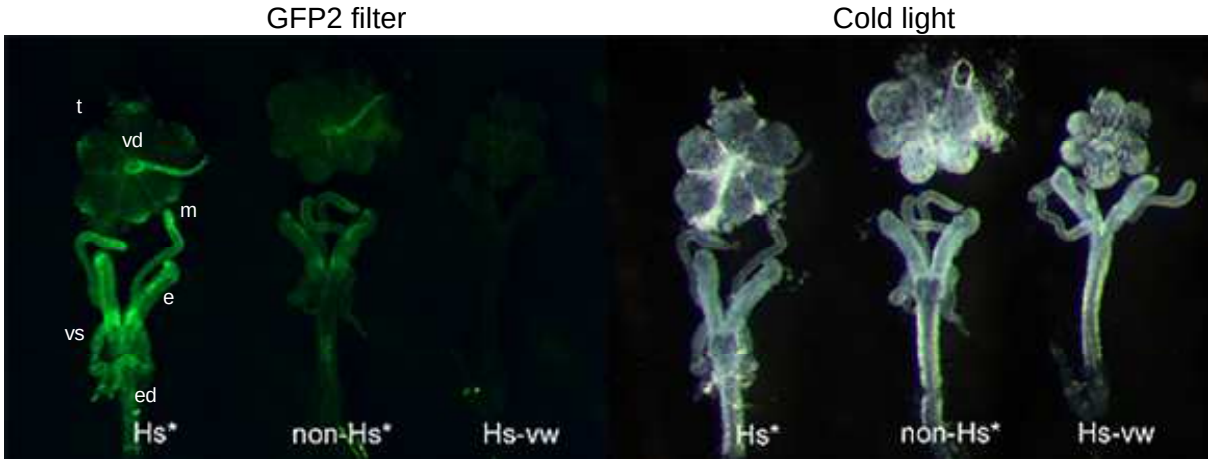


Figure 3-9 Male reproductive organs.
Heat-shocked transgenic (Hs*) and *vermilion*^{white} pupae (Hs-vw) with non-heat-shocked transgenic one (non-Hs*) were dissected. Male testes (t) and two pairs of accessory glands, ectadenia (e) and mesadenia (m) specifically reacted to the heat shock. *hsp70* promoter response was evident in testicular follicles in apical cells and in *vasa deferentia* (vd) that is an extended part of these follicles continuing to *vesicula seminalis* (vs) and joining ejaculatory duct (ed). *Vas deferens* and *ectadenia* parts possess slight constitutive *hsp70*-EGFP expression in non-Hs*. Note that only one testis, separated from *vesicula seminalis* joining, is shown in the picture. Line D is presented.

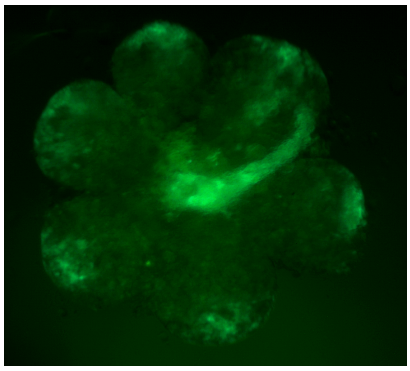


Figure 3-10 *Tribolium* testis.
Six testicular follicles are apparent. Induced EGFP expression was found in apically placed cells of testicular follicles and in place, where they connect together, entering the *vas deferens*.

3.1.4.2 *Drosophila hsp70* response to the heat shock in *Tribolium* brain and gut

As reproductive organs responded to the heat shock, the *hsp70* promoter was expected to be inducible also in other *Tribolium* tissues. In following, *Tribolium* brain and gut of pupal stage (transgenic line D) were investigated (Fig. 3-11, Fig. 3-12). A strong constitutive EGFP expression (Fig. 3-11, non-Hs*, A), but weak induced EGFP expression (Fig. 3-11, Hs*, A) was visible in proto-, deuto- and trito-cerebral ganglion of pupal brain. This is consistent with above described characteristic of line D. Intense 3xP3-DsRed marker was expressed in the eyes of transgenic pupae (Fig. 3-11, B) strongly transmitting under GFP2 filter (Fig. 3-11, A). Horn et al. (2000) demonstrated that 3xP3 promoter is expressed in *Drosophila* eyes, central nervous system (CNS), peripheral nervous system (PNS) and other non-nervous system parts. Here, in the brain of transgenic *T. castaneum*, two small ganglions in proto-cerebral part were visible (Fig. 3-11, A and B).

Furthermore, the same pupae (transgenic line D) were dissected to evaluate the heat-shock response of the digestive system (Fig. 3-12). Induced EGFP expression was visible in hind gut and also in mid gut. However, induced EGFP marker was decreased due to strong autofluorescence in mesenteron (Fig. 3-12, non-Hs*, A). Constitutive and induced EGFP expression was almost undistinguishable in Malpighian tubuli (compare Hs* and non-Hs* in A, Fig. 3-12).

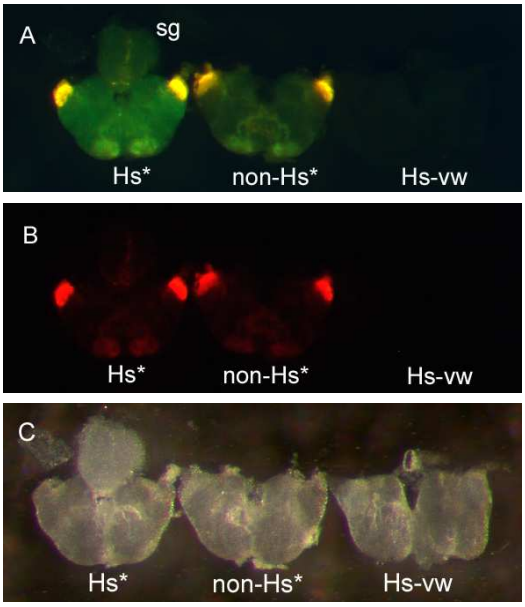


Figure 3-11 *Tribolium* brain.
Heat-shocked transgenic pupal brain (Hs*) displayed a specific heat-shock response in the whole brain compared to non-heat-shocked transgenic brain (non-Hs*) and heat-shocked *vermilion*^{white} brain (Hs-vw). Additional subesophageal ganglion (sg) is shown in the case of Hs*. 3xP3-DsRed transformation marker corresponds to the eyes and optical nerves of CNS in pupal brain. Transgenic line D is shown. A, GFP2 filter; B, DsRed filter; C, cold light.

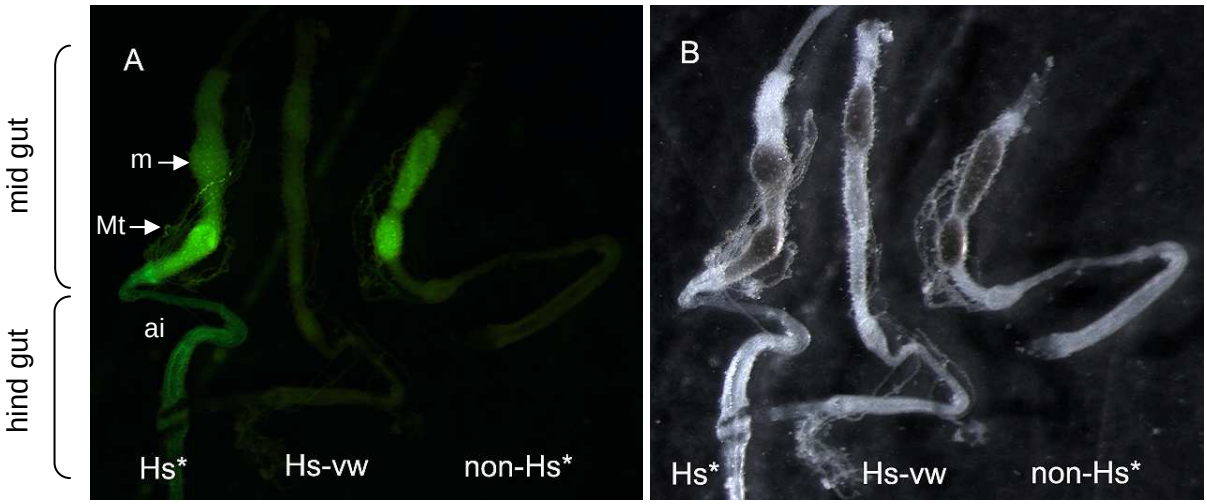


Figure 3-12 *Tribolium* digestive system.
Transgenic pupae, heat-shocked (Hs*) and non-heat-shocked (non-Hs*), were dissected and compared for *hsp70* response to the heat-shock with *vermilion*^{white} mutant (Hs-vw). Transgenic line D was analyzed. A, GFP2 filter; B, cold light. Mt = *Malpighian tubuli*, m = *mesenteron*, ai = *anterior intestine*.

3.2 DEVELOPMENT OF ASSAY SYSTEMS FOR THE EVALUATION OF BINARY EXPRESSION SYSTEMS

In the first part (3.2.1), universal constructs for Gal4/*UAS*, LexA/*(LL)$_4$* and the tetracycline-controlled systems were created and introduced into *D. melanogaster*. This allowed not only to test constructs and systems for their functionality, but also to compare among the binary expression systems in this species (3.2.2). The third part (3.2.3) will address their introduction into the red flour beetle, *Tribolium castaneum* and its preliminary evaluation of their applicability there.

3.2.1 Germ-line transformation of *D. melanogaster*

To test whether universal constructs (Material and methods, 2.1.2) were functional, firstly, transgenic flies were generated (Tab. 3-2). At least three independent lines on different chromosomes were obtained for each construct. In the case of Gal4VP16, however, only one line was obtained. To generate other independent lines, another injection round of this construct was performed, but had failed (ca 600 eggs were injected, 120 of those hatched, 19 male crosses were set up). This difficulty to generate transgenic lines was observed only with this construct. The Gal4VP16 line was used to remobilize the construct to other genomic site. Remobilization efficiency was around 32%, which is comparatively little to the regular 88% (Horn et al. 2003). Other two independent lines were set up.

Table 3-2 Transformation efficiencies of various components of analyzed binary expression systems

System variant	No. of eggs injected	No. of hatched larvae	No. of male crosses	No. of sterile crosses	No. of transgenic lines
Gal4	Flies provided by M. Klingler				
Gal4Δ	ca 600	ca 200 (33.3%)	25 (12.5%)	3 (12%)	4 (18.2%)
Gal4VP16	ca 540	ca 45 (8.3%)	17 (37.7%)	3 (17.6%)	1 (7.1%)
UAST	ca 780	ca 200 (25.6%)	62 (31%)	19 (30.6%)	7 (16.3%)
UASp	Flies provided by C. Horn				
LexA	ca 450	29 (6.4%)	17 (58.6%)	5 (29.4%)	3 (25%)
(LL)$_4$	ca 450	75 (16.6%)	43 (57.3%)	13 (30.2%)	7 (35%)
tTA	ca 600	ca 200 (33.3%)	31 (15.5%)	5 (16.1%)	10 (38.5%)
sctTA	ca 900	ca 200 (22.2%)	79 (39.5%)	20 (25.3%)	10 (16.9%)
scrtTA	ca 700	ca 330 (47.1%)	99 (30%)	15 (15.2%)	6 (7.1%)
TRE	Flies provided by C. Horn				

Transformation efficiencies = No. of transgenic lines/No. fertile crosses; the percentage of hatched larvae = No. of hatched larvae/No. of injected eggs; the percentage of sterile crosses = No. of sterile crosses/No. of male crosses; other percentages are related to No. of hatched larvae. Gal4VP16 construct injection resulted in one line. To have three independent lines, this line was used for a remobilization of the construct to other genomic sites.

3.2.2 Analyses of binary expression systems in *D. melanogaster*

A reproducible kinetic analysis of ß-galactosidase reporter was established in *D. melanogaster* (see Material and methods, 2.6.2). It allowed testing and comparing: (i) independent lines within a binary expression system, (ii) binary expression system variants and (iii) different binary expression systems.

3.2.2.1 Analysis of Gal4/*UAS* system and its variants

Gal4, Gal4Δ and Gal4VP16 variants and *UAST* or *UASp* variants were combined. Six system variant combinations were tested and three independent lines for each, the transactivator and the responder, were used. All these measurements were performed independently on two different days to check for reproducibility. Based on ß-gal kinetics, Gal4Δ transactivator showed always the highest potential to activate the reporter gene, *lacZ*, expression than Gal4VP16 or original Gal4 one. Moreover, all transactivators worked better with *UAST* responder than with *UASp*.

The minimal and maximal values of each system variant combination were evaluated according to mOD/min (Tab. 3-3). Graphical arrangements for all set ups are shown in Appendix, part A. In the case of Gal4Δ/*UAST*, maximal mOD/min was observed for Gal4Δ-A2/*UAST*-R1 combination in the first measurement while for Gal4Δ-A2/*UAST*-R3 in the second one. This disagreement was likely due to a failure caused by a measurement of very high ß-gal enzymatic activity. The same independent line Gal4Δ-A2 had though the highest potential to drive responder lines. Although minimal mOD/min was obtained in the case of different combinations Gal4Δ-A2/*UASp*-R3 and Gal4Δ-A3/*UASp*-R3, it is visible that Gal4Δ-A2 and Gal4Δ-A3 transactivator are similarly potent to drive the ß-gal responder.

In the case of Gal4VP16/*UAS*, only one Gal4VP16-A1 line was functional. Other two lines obtained by the construct remobilization did not perform any ß-gal enzymatic activity in combination with three independent responder lines. It could have been due to a position effect. A disagreement between the first and the second measurement in the case of Gal4VP16/*UAST* was likely due to the fact that not enough flies were available for three independent homogenate samples and difficulties with reproducibility of three independent samples were observed. Because mOD/min values were influenced by these problems and results were based on one line Gal4VP16-A1, thus, the evaluation of Gal4VP16/*UAS* system should be taken as a pilot analysis.

Table 3-3 ß-gal kinetics of Gal4/*UAS* system and its variants

System variant	Measurement	Min mOD/min	AxRx combination	Max mOD/min	AxRx combination	Maen mOD/min	± s.d.
Gal4/*UAST*	1st	5.50	A3R2	11.38	A1R3		
	2nd	4.85	A3R2	11.45	A1R3		
	Overall					**8.36**	**1.79**
Gal4/*UASp*	1st	4.97	A2R3	6.52	A3R1		
	2nd	4.34	A2R3	5.57	A3R1		
	Overall					**5.34**	**0.63**
Ga4Δ/*UAST*	1st	8.23	A3R1	28.41	A2R1(A2R2)		
	2nd	9.45	A3R1	36.01(30)	A2R3(A2R1)		
	Overall					**20.81**	**8.19**
Gal4Δ/*UASp*	1st	9.53 (9.67)	A2R3 (A3R3)	18.77	A1R1		
	2nd	10.89 (11.14)	A3R3 (A2R3)	19.56	A1R1		
	Overall					**14.61**	**3.19**
Gal4VP16/*UAST*	1st	12.56	A1R1	16.32	A1R3		
	2nd	14.99	A1R3	16.24	A1R2		
	Overall					**14.59**	**1.6**
Gal4VP16/*UASp*	1st	7.01	A1R3	9.66	A1R2		
	2nd	9.29	A1R3	13.53	A1R2		
	Overall					**10.06**	**2.27**

All possible combinations of Gal4 and *UAS* variants were measured for the ß-galactosidase kinetics. Minimal and maximal mOD/min values of AxRx combinations corresponded to independent line combinations within a Gal4/*UAS* system variant (A= a transactivator, R= a responder and x = a number of independent line; see Tab. 2-3 in material and methods). In the case of Gal4VP16 transactivator, however, only one line Gal4VP16-A1 was functional. Mean mOD/min were calculated over all kinetic measurements within one Gal4/*UAS* variant combination (in bold letters). Standard deviation (± s.d.) is indicated.

3.2.2.2 Analysis of LexA/*(LL)$_4$* system

In comparison to other analyzed systems, different collection times and distinct fly aging at 25℃ room or 18℃ room were carried out for LexA /*(LL)$_4$* system (see Material and methods, part 2.6.2, animal crosses). In spite of that, obtained mOD/min values were still comparable (Appendix, part B), suggesting robust reproducibility of the established kinetic approach (Material and methods, part 2.6.2). When compared to the second and the third measurements, results of the first measurement pointed out that the eyes of ca. six days old flies (kept in 25℃) already contained such amount of ß-gal reporter, whose enzymatic activity did not much change with additional fly aging (i.e. with a potential accumulation of ß-gal reporter protein amount in the eyes). Moreover, LexA transactivator was capable of activating the reporter gene expression in 25℃ as well as in 18℃. Based on obt ained results, however, it is not clear whether comparable ß-gal enzymatic activity will be still observed in *Drosophila* eye extracts of flies that would be kept ca. six days in 18℃.

Tab. 3-4 shows that LexA-A3 transactivator performed the lowest potential to drive the ß-gal reporter in the third measurement unlike LexA-A2, which displayed the lowest potential in two previous measurements. It is clear that R1 responder worked the worst in combination with LexA transactivator while R2 one worked the best. It could explain why LexA-A3 displayed the minimal mOD/min in the third measurement in combination with this responder unlike LexA-A2. Graphs are presented in Appendix, part B.

Table 3-4 ß-galactosidase kinetics of LexA/*(LL)₄* system

System	Measurement	Min mOD/min	AxRx combination	Max mOD/min	AxRx combination	Mean mOD/min	±s.d.
LexA/*(LL)₄*	1st	1.06	A2R1	3.44	A2R2		
	2nd	2.09	A2R1	3.30	A2R2		
	3rd	1.91(2.24)	A3R1 (A2R1)	2.78	A2R2		
	Overall					**2.34**	**0.28**

Three measurements were done for LexA/*(LL)₄* system. Homogenate samples were prepared from heads of flies collected: 10h (1st measurement), 24h (2nd one) and 21h (3rd one) after fly eclosure. Minimal and maximal values of AxRx combination were indicated. AxRx represents a combination of three independent lines. A = a transactivator, R = a responder (see Tab. 2-3 in Material and methods). Mean mOD/min was calculated over all mOD/min values of this system. Standard deviation is included (± s.d.).

3.2.2.3 Analysis of the tetracycline-controlled systems

tTA, sctTA, scrtTA transactivator variants were combined with their responsive element *TRE*. Three different variant combinations tTA/*TRE*, sctTA/*TRE* and scrtTA/*TRE* were evaluated. Three independent lines for each transactivator and *TRE* responder were analyzed. To activate scrtTA/*TRE* system, fly food was enriched with tetracycline antibiotic (100 µg/ml). To control a possible constitutive activity of this system, controls were carried out on the food without tetracycline as well. Minimal and maximal mOD/min values of each system variant are shown in Tab. 3-5. Graphs are presented in Appendix, part C.

None of the system variants performed mOD/min values, which differed from those of *TRE* responder controls (see Appendix, part C) or from w^- controls. No significant difference between mOD/min values of scrtTA/*TRE* (+Tc) and scrtTA/*TRE* (-Tc) was observed. Based on that, no induced β-gal enzymatic activity was detected by this approach. To check whether this was due to limited number of analyzed fly heads (three male and three female heads), thirty heads were taken for another β-gal analysis of tTA/*TRE* and sctTA/*TRE* system. No difference from the controls was observed again (data not shown). To ensure that all obtained mOD/min values for system variants were not artefacts; pure β-galactosidase enzyme was used as a positive control (data not shown).

Table 3-5 ß-galactosidase kinetics of tetracycline-controlled system

System variant	Measurement	Min mOD/min	AxRx combination	Max mOD/min	AxRx combination	Mean mOD/min	± s.d.
tTA/*TRE*	1st	0.33	A1R1	0.76	A2R3		
	2nd	0.43	A1R1	0.80	A2R3		
	Overall					**0.59**	**0.14**
sctTA/*TRE*	1st	0.48	A2R2	0.79	A2R3		
	2nd	0.53	A2R2	0.88	A2R1		
	Overall					**0.67**	**0.18**
scrtTA/*TRE*	1st	0.35	A3R1	0.58	A1R3		
+Tc	2nd	0.36	A3R1	0.60	A2R3		
	Overall					**0.44**	**0.08**
scrtTA/*TRE*	1st	0.27	A3R2	0.51	A1R3		
-Tc	2nd	0.31	A3R2	0.50	A1R3		
	Overall					**0.38**	**0.07**

Two measurements were done for each tetracycline-controlled system variant. mOD/min values were calculated within one system variant. Minimal and maximal values were chosen and AR combination to which these values belonged is shown. AR represents a combination of three independent lines within one system variant. A = a transactivator; R = a responder; a suffix numbers at A and R correspond to independent lines tested (the overview was shown in Tab. 2-3 in material and methods). Mean mOD/min value was calculated over all mOD/min values within one system variant. Standard deviation is indicated (± s.d.). +Tc, experiment carried out on tetracycline enriched fly food, -Tc, standard fly food without tetracycline.

tTA/*TRE* and sctTA/*TRE* systems were tested for a direct presence of the β-gal protein in head extracts. No specific signal for this protein was observed (data not shown). A presence of sctTA and scrtTA transactivator protein was detected in head extracts (Fig. 3-13). Immunoblotting against a protein of tTA transactivator was negative (data not shown). This was repeated with doubled number of fly heads per homogenate sample. No positive signal was observed again (data not shown).

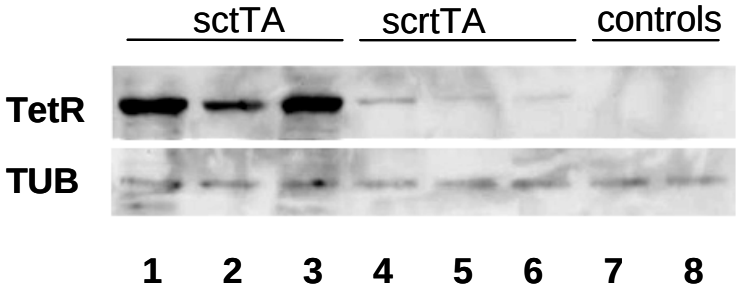


Figure 3-13 sctTA and scrtTA transactivators under 3xP3 promoter were present in *Drosophila* head extracts. Immunoblotting against TetR, DNA-binding domain of sctTA and scrtTA is shown. Fly heads containing sctTA/*TRE* and scrtTA/*TRE* system (-Tc) were analyzed. High protein presence was detected for sctTA-A1, sctTA-A2, sctTA-A3 lines (lane 1, 2, 3, respectively). Much lower protein level was observed for scrtTA-A1, scrtTA-A2 and scrtTA-A3 lines (lane 4, 5, 6, respectively). Lane 7,*TRE*-R1 responder control (-Tc); lane 8, *w-* control (-Tc). Re-probing with anti-α-tubulin (TUB) was used as a loading control. (-Tc), flies were kept on standard food without tetracycline.

3.2.2.4 Comparison among Gal4/*UAS*, LexA/*(LL)$_4$* and tetracycline-controlled systems in *Drosophila melanogaster*

Binary expression systems were analyzed in previous parts of 3.2.2. To compare among these systems, their mean mOD/min values (Tab. 3-6) were plotted into a graph (Fig. 3-14). To show a variability within one system variant (e.g. Gal4Δ/*UASp*), standard deviations were indicated. Results suggested that these systems work differently in *D. melanogaster*. Gal4/*UAS* system was found to be the best working one. LexA/*(LL)$_4$* system performed only moderate level. The worst results were observed for the tetracycline-controlled system.

The mOD/min values of their responder controls as well as *w*$^-$ controls were included. An overview of these mOD/min values is in Tab. 3-6. Mean mOD/min value of a system variant was corrected according to mean mOD/min value of its responder control (Tab. 3-7). Corrected values were plotted to a graph (Fig. 3-15). The order of the system variants, which corresponded to their working efficiencies in photoreceptors of *D. melanogaster*, did not change. The tetracycline-controlled system showed no working efficiency.

Table 3-6 Overview of mOD/min values

System variant	Mean mOD/min	± s.d.	Responder controls	Mean mOD/min	± s.d.	Responder controls	Mean mOD/min overall	± s.d.
Gal4Δ/*UAST*	20.806	8.196	*UAST*	0.580	0.053	***UAST***	0.466	0.111
Gal4Δ/*UASp*	14.613	3.191	*UASp*	0.492	0.025	***UASp***	0.417	0.083
Gal4VP16/*UAST*	14.598	1.604	*UAST*	0.351	0.080	***(LL)$_4$***	0.610	0.134
Gal4VP16/*UASp*	10.062	2.268	*UASp*	0.319	0.049	***TRE* (+Tc)**	0.403	0.063
Gal4/*UAST*	8.361	1.796	*UAST*	0.468	0.042	***TRE* (-Tc)**	0.558	0.173
Gal4/*UASp*	5.337	0.632	*UASp*	0.443	0.038	**w- (+Tc)**	0.415	0.011
LexA/*(LL)$_4$*	2.479	0.517	*(LL)$_4$*	0.610	0.134	**w- (-Tc)**	0.478	0.185
tTA/*TRE*	0.588	0.143	*TRE*	0.593	0.101			
sctTA/*TRE*	0.669	0.175	*TRE*	0.700	0.148			
scrtTA/*TRE* (+Tc)	0.442	0.076	*TRE* (+Tc)	0.403	0.063			
scrtTA/*TRE* (-Tc)	0.381	0.070	*TRE* (-Tc)	0.388	0.069			

Mean mOD/min value of a system variant was calculated over mOD/min values of nine possible combinations. Nine combinations resulted from crosses of three independent lines for the transactivator and the responder. These nine combinations were measured twice. mOD/min of each combination was based on three independent measurements. In the case of Gal4VP16/*UAS* system, only combinations of functional line A1 with three independent responder lines were taken for the evaluation of this system. Moreover, for each system variant mOD/min value of its responder control was calculated. This responder control was calculated over mOD/min values of three independent responder lines. mOD/min of one independent combination was based on three measurements. Mean mOD/min value of responder (right part of table) was a value of a responder control estimated over all measurements, where this type of the responder was used as a control. Mean responder controls and *w*- controls were not subtracted from values of system variants. In bold letters are indicated values, which were plotted in the graph (Fig. 3-14).

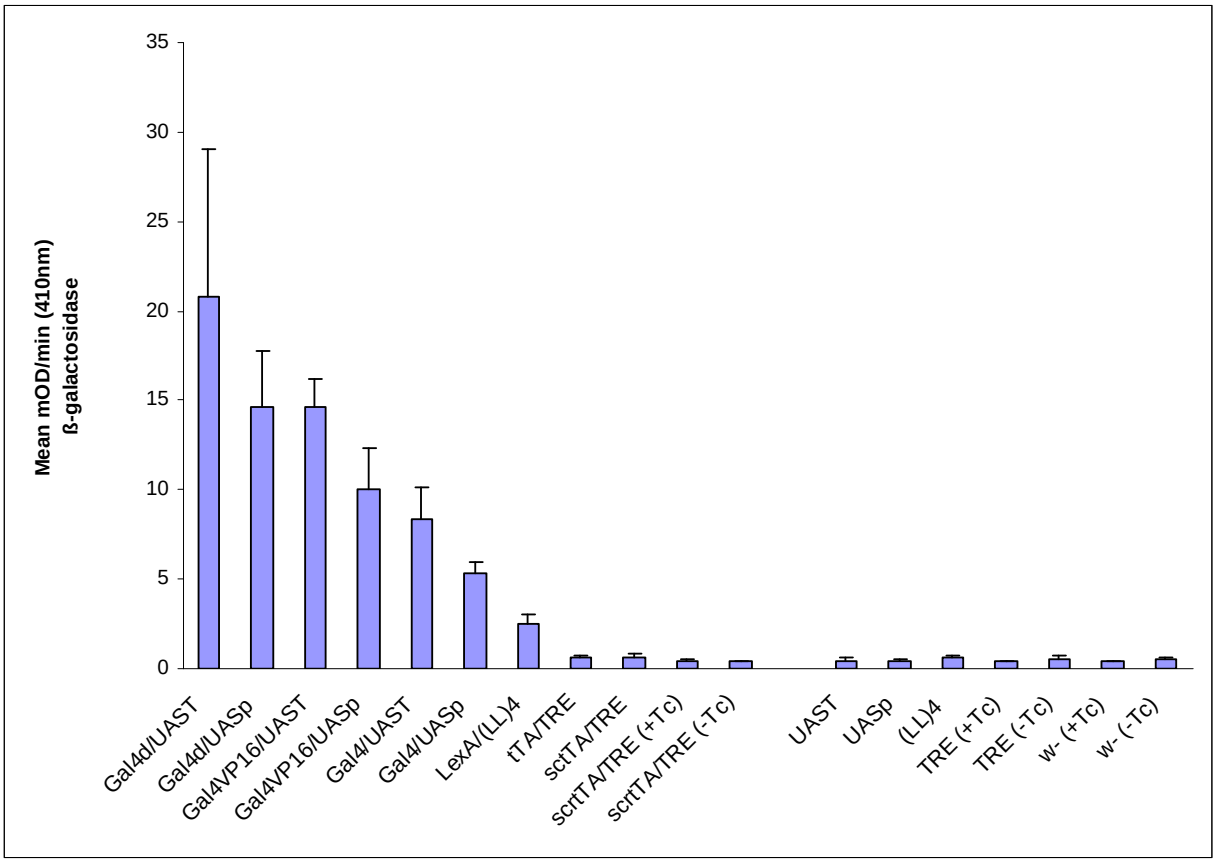


Figure 3-14 Comparison among analyzed binary expression systems.
Gal4/*UAS*, LexA/*(LL)*$_4$ and tetracycline-controlled systems are presented on x axis. Controls for responders as well as *w-* flies were included. Flies for *TRE* and *w-* controls were fed on standard fly food (-Tc) as well as on enriched food with tetracycline antibiotic (+Tc). Columns represent mean mOD/min based on β-gal enzymatic activity. This activity was measured in one interval minute for 90 min at 410 nm. Standard deviations are signed as a bar on each column. The controls were not subtracted from system values. Gal4d = Gal4Δ.

Table 3-7 Mean mOD/min values of system variants and their corrections

System variant	Original mean mOD/min	± s.d.	Corrected mean mOD/min
Gal4Δ/*UAST*	20.806	8.196	20.226
Gal4Δ/*UASp*	14.613	3.191	14.121
Gal4VP16/*UAST*	14.598	1.604	14.247
Gal4VP16/*UASp*	10.062	2.268	9.743
Gal4/*UAST*	8.361	1.796	7.893
Gal4/*UASp*	5.337	0.632	4.894
LexA/*(LL)*$_4$	2.479	0.517	1.869
tTA/*TRE*	0.588	0.143	0
sctTA/*TRE*	0.669	0.175	0
scrtTA/*TRE* (+Tc)	0.442	0.076	0
scrtTA/*TRE* (-Tc)	0.381	0.070	0

Mean mOD/min value of a responder control was subtracted from a mean mOD/min value of the system variant, where this responder was used. The “0” mOD/min value indicates that the responder control of the tetracycline-controlled system was greater than the value of system itself.

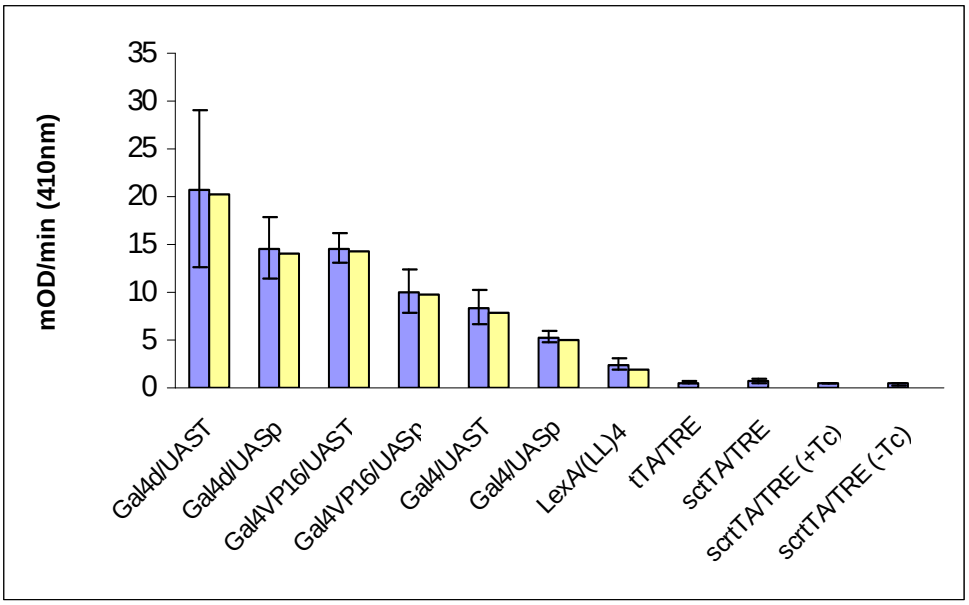


Figure 3-15 Comparison among the binary expression systems.
Each system variant is represented by two columns. The first column was based on original mean mOD/min value of β-galactosidase enzymatic activity, which was measured within 90 min at 410 nm. Standard deviation was indicated as a ± bar. In other column, corrected mOD/min is shown. This value was obtained when a mOD/min values of a responder control was subtracted from the original mean mOD/min value of a system variant, where this responder was used. In the case of the tetracycline-controlled systems, such corrections resulted in null values for all system variants. Ga4d = Gal4Δ.

Gal4/*UAS* and LexA/*(LL)*$_4$ binary expression systems were proven to be functional. The tetracycline-controlled system, however, performed the ß-gal enzymatic activity comparable to the responder and w^- control. Therefore, the transactivator constructs were sequenced and confirmed for their correctness. As two out of three independent responder lines (*TRE*-R1 and *TRE*-R2) were shown to function by *in situ* hybridization (J. Schinko, personal comm.), it excluded that the *TRE* construct lines were the reason for induced ß-gal level.

3.2.3 Introduction of binary expression systems into the germline of *T. castaneum*

Three mostly used binary expression systems placed in *piggyBac* backbone were compared in previous part in *D. melanogaster* (3.2.2.4). The usage of the broad-range *piggyBac* transposable elements and the universal 3xP3 promoter makes it feasible to introduce them into the germline of non-drosophilid species. Their introduction into the red flour beetle, *T. castaneum* and preliminary tests will be addressed in the following parts.

3.2.3.1 Germ-line transformation of *T. castaneum*

Three transgenic lines were successfully set up for: (i) Gal4Δ and Gal4VP16 transactivator; (ii) *UAST* responder. Low transformation efficiencies were observed (Tab. 3-8). The highest was obtained for Gal4VP16 (4.6%) and the lowest for Gal4Δ (1.3%). *UAST* responder lines were generated with 1.8% transformation efficiency. The segregation of used transformation markers in next generations suggested likely single insertions (or insertions on one chromosome). Transgenic beetles representing both transactivator lines and *UAST* line are shown in Fig. 3-16. Interestingly, only a few photoreceptors that express transformation marker were observed in a few lines of both types of Gal4 transactivator as well as *UAST* responder. This 'dotted' pattern was heritable.

Table 3-8 Generation of transgenic lines and transformation efficiencies in *T. castaneum*

Construct in *piggyBac*	No. of injected eggs	No. of hatched larvae	No. of all crosses	No. of sterile crosses	No. of transg. lines	Transg. line	No. of insertions	Line available
3xP3-EYFP;3xP3-Gal4VP16	1813	198 (10.9%)	133 (67.2%)	4 (3%)	6 (4.6%)	A	single	yes
						B	more*	no
						C†		no
						D	single	yes
						E†		no
						F†		no
3xP3-EYFP;3xP3-Gal4Δ	1953	159 (8.1%)	78 (49.1%)	2 (2.6%)	1 (1.3%)	A1	single	yes
						A2	more	yes
						A3	single	yes
3xP3-DsRed;*UAST*-lacZ	2337	364 (15.6%)	231 (63.5%)	14 (6.1%)	4 (1.8%)	A	single	yes
						B1	more	yes
						B2	single	yes
						C	single*	no
						D	single*	no

The efficiency of hatched larvae = No. of hatched larvae/No. of injected eggs. The efficiency of sterile crosses = No. of sterile crosses/No. all crosses; the percentage of all crosses was related to the No. of hatched larvae. All male and female pupae, which developed from injected eggs, were crossed against *vermilion*^{white} mutant of the opposite gender. The transformation efficiency of transgenic line = No. of transgenic lines/No. of fertile crosses. *, transgenic lines were lost when investigated for the number of insertions. Transg., transgenic line; †, transgenic animals, which were found, died either as pupae or were sterile.

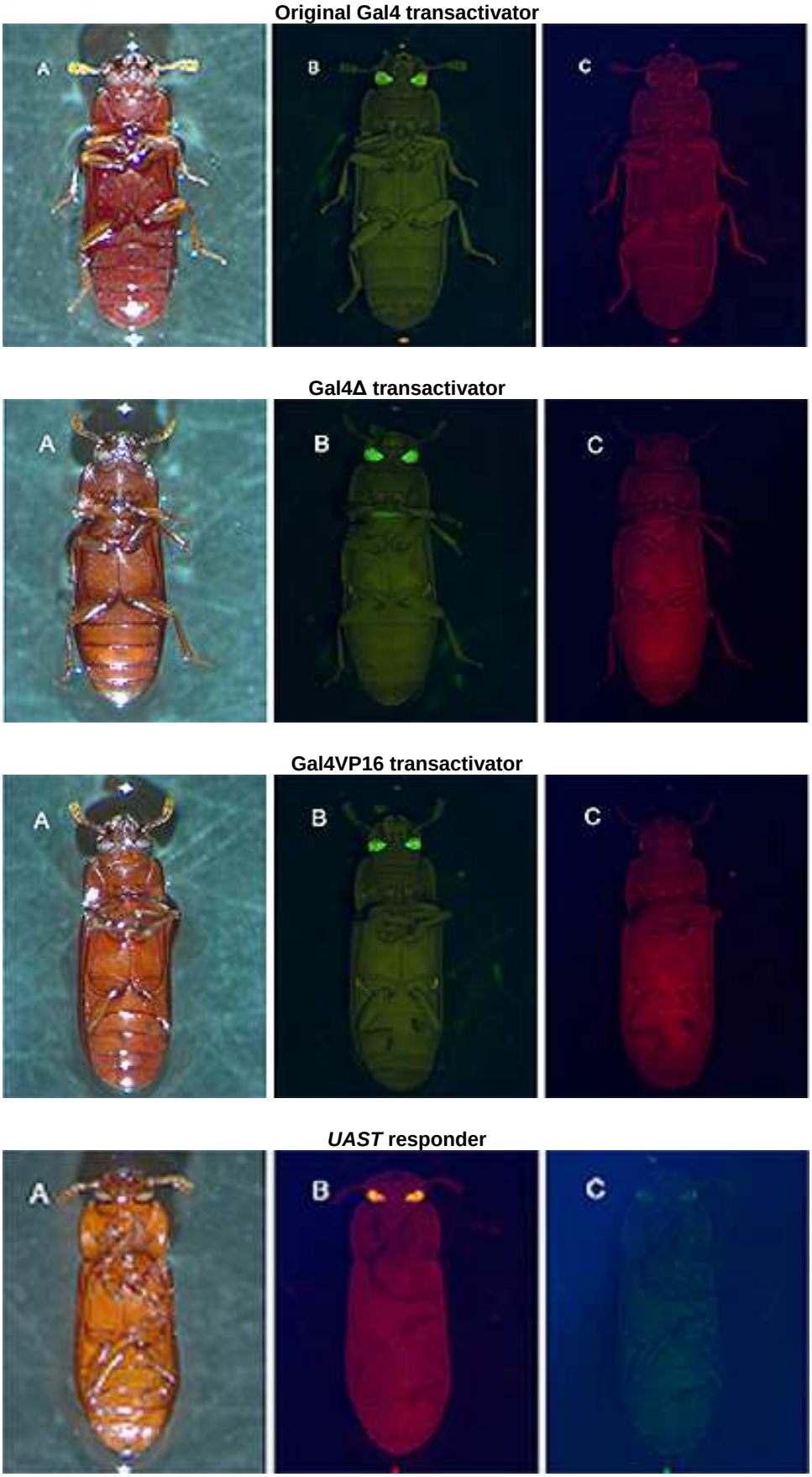


Figure 3-16 Transgenic lines in *T. castaneum*.
Transactivators: A, cold light, B, GFP2 filter, C, DsRed filter. Responder: A, cold light, B, DsRed filter, C, YellowGFP filter.

3.2.3.2 Preliminary tests and the evaluation of Gal4/*UAS* system in *T. castaneum*

To determine whether the Gal4/*UAS* system works in *T. castaneum*, available Gal4VP16 (line A) and Gal4Δ (line A3) transactivator lines were crossed against *UAST* (line B2) responder line. Beetles with both components of Gal4/*UAS* system were recognized according to transformation markers (Fig. 3-17).

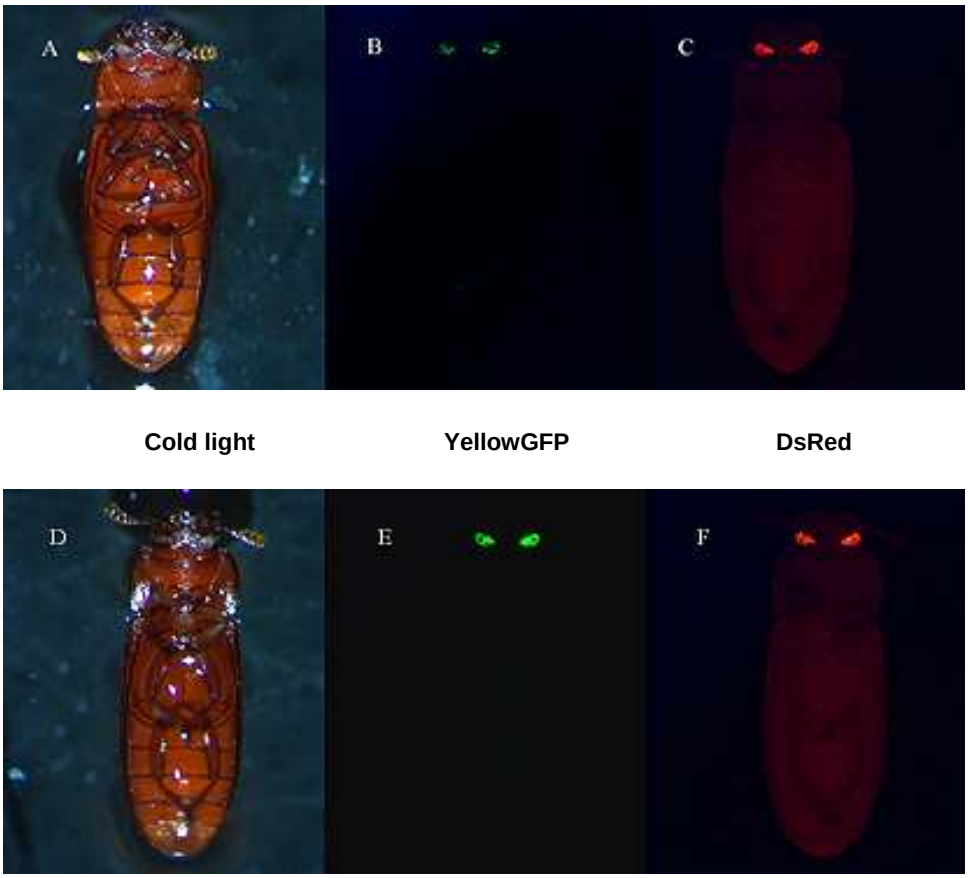


Figure 3-17 Gal4VP16/*UAST* system (A-C). Gal4Δ/*UAST* system (D-F).
Both, the transactivator (3xP3-EYFP; 3xP3-Gal4VP16) and the responder (3xP3-DsRed; *UAST*-lacZ) in *piggyBac* backbone were present in the beetle. Gal4VP16 transactivator shows the weaker expression of transformation marker than Gal4Δ. YellowGFP and DsRed = filters; exposure time = 25 s.

X-gal assay

In *Drosophila*, X-gal assay is very common to stain β-gal reporter protein without high background (e.g. Greenspan 1995). This method enables easy-to-stain *Drosophila* inner parts. It was expected that 3xP3-Gal4Δ/*UAST*-lacZ should cause specific blue colour in the eyes and consequent reduction of the intensity of transformation markers, 3xP3-EYFP and 3xP3-DsRed1, in *D. melanogaster* and *T. castaneum*. For this purpose, improved X-gal assay with DMSO was used, allowing the X-gal penetration directly towards tissues in the place of a disruption.

Tribolium pupae were used to easily identify the blue colour, which would be difficult in adults due to their cuticle pigmentation. Pupae were raised in 25℃ incubator. As a positive control served adults of *D. melanogaster*, containing functional LexA/*(LL)*$_4$ system. These flies were kept at 18℃ room over one week. Expectations postulated above were fulfilled in *D. melanogaster*, but not in *T. castaneum.* Surprisingly, unspecific background of blue colouration appeared in *vermilion*^{white} mutants as well as transgenic pupae of *T. castaneum* (Fig. 3-18).

β-galactosidase kinetics and immunoblotting

Beetles were kept at least 10 days at 33℃ incubat or, so that there was enough time for the reporter gene, *lacZ*, expression and its protein accumulation (β-gal). Such high temperature should not harm yeast Gal4 protein that performs the best activity around 29℃ (e.g. Tower 2000). Three heads were taken per sample for β-gal kinetics. Preliminary tests revealed no significant activity of this reporter (data not shown). To eliminate a possibility of a low β-gal protein level in the sample from three heads, a direct test for the presence of this protein was performed by immunoblotting. For this purpose, six heads (Gal4VP16/*UAST*) and ten heads (Gal4Δ/*UAST*) were analyzed. As *Tribolium* negative control was used original *vermilion*^{white} mutant strain. As a positive control served LexAGAD/*(LL)*$_4$ system, which was proven to be functional in *D. melanogaster* (part 3.2.2.2). As *Drosophila* negative control, *w*$^{-}$ flies (*D. melanogaster)* were used

No signal for β-gal protein was observed for Gal4VP16/*UAST* and Gal4Δ/*UAST* system in *T. castaneum* (Fig. 3-19). Strong signal was detected for LexAGAD/*(LL)*$_4$ positive control. Notably weaker signal appeared also in *(LL)*$_4$ responder, suggesting a potential constitutive expression of this analyzed line M17A.III. This responder line was not used for kinetic analysis (Material and methods, part 2.6.2.1).

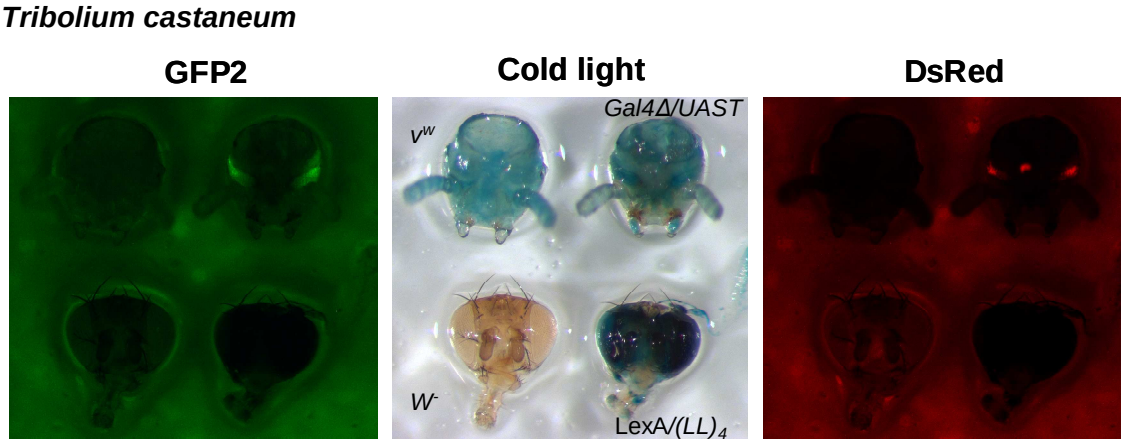


Figure 3-18 X-gal assay by using DMSO.
Tribolium pupal heads and adult heads of *D. melanogaster* were stained for β-gal activity by using X-gal assay (in DMSO). This caused apparent reduction of transformation marker detectibility in the eyes (3xP3 promoter) in transgenic heads of *D. melanogaster*, containing LexA/*(LL)*$_4$ system. No visible blue colour appeared in *w-* mutants. By contrast, neither eyes of *Tribolium* pupal heads containing Gal4Δ/*UAST* system nor pupal eyes of *vermilion*^{white} mutant displayed specific blue colouration. Surprisingly, unspecific blue colouration in *Tribolium* pupal heads appeared unlike in *Drosophila*.

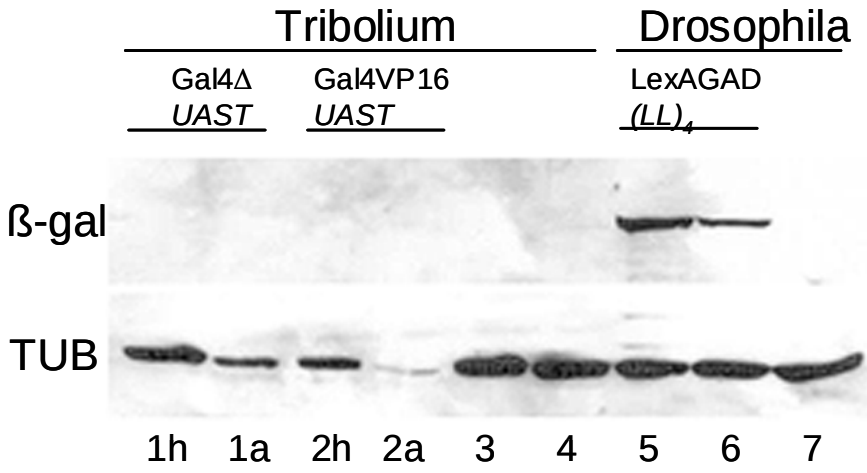


Figure 3-19 Variants of Gal4/*UAS* system did not show any activity in *Tribolium castaneum*.
Head extracts were analyzed for presence of β-galactosidase (β-gal). As positive control served the extract of *D. melanogaster* heads, containing functional LexAGAD/*(LL)*$_4$ system (lane 5). *(LL)*$_4$ responder control (lane 6) surprisingly showed strong leakiness of the reporter construct of the analyzed line M17A.III. Heads of *D. melanogaster w-* flies were used as negative control, indicating no β-gal presence (lane 7). In all *Drosophila* lanes 5 heads extracts were loaded. In *Tribolium*, no reporter protein was detected for Gal4Δ/*UAST* (lane 1h and 1a) and Gal4VP16/*UAST* (lane 2h and 2a). *UAST* responder control (lane 3) and *vermilion*^{white} mutant control (lane 4) did not give any signal for β-gal as well. *Tribolium* lanes were loaded with 10 head extracts, except of 1a and 2a, where 1 abdomen was analyzed. And lane 2h corresponds to 6 head extract. "h" = head and "a" = abdomen. Additionally, re-probing with anti-α-tubulin (TUB) was performed as a loading control.

3.3 ENGINEERING OF CHROMOSOMAL REARRANGEMENTS BY USING FLP/*FRT* MEDIATED RECOMBINATION AND NON-P-ELEMENTS IN *Drosophila melanogaster*

Götschel (2003) established several *FRT* lines in *piggyBac* and *Hermes* backbone in *D. melanogaster*. The chromosomal insertion site of each *FRT* line was identified by using inverse PCR and their cytological localization as well as their *FRT* site orientation was determined. It provided the set up to create of defined inversions, deletions and duplications. Thus, his work is the prerequisite for the following experiments, which were done with his cooperation.

3.3.1 Confirmation of defined inversions

Prior to the usage of established inversions for further experiments (in part 3.3.2 and 3.3.3), tests were carried out whether the fluorescent marker switch, which was the consequence of the FLP/*FRT* mediated recombination between *FRT* target sites of two *FRT* constructs (see Material and methods, Fig. 2-7), corresponded to the inversion of defined chromosomal region. For this purpose, all four independent lines (#1, #2, #3, #4) of *X* chromosomal inversion (F1/F1) were analyzed. Based on Giemsa staining of polytene chromosomes, defined chromosomal region of this *X*-linked inversion between cytoband 13D2 and 19E6 (Fig. 3-20) was found to be inverted in all analyzed lines. The inverted chromosomal region of the line #2 is shown in Fig. 3-21. It fulfilled expectations and suggested the fluorescent marker switch as a reliable marker for the identification of defined chromosomal rearrangements in *D. melanogaster*.

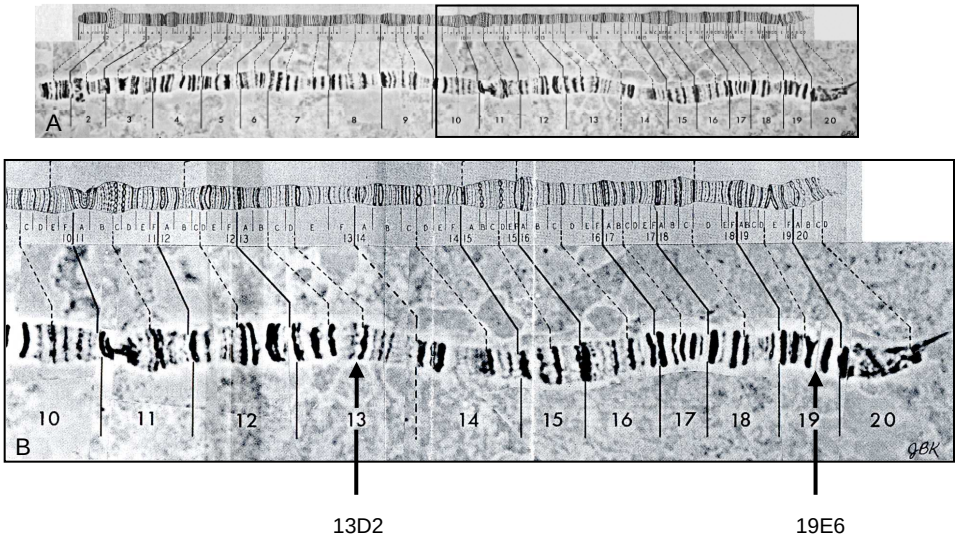


Figure 3-20 Cytology of *Drosophila* wild type *X* chromosome.
The magnification of the box (black line, picture A), selecting the part of *X* chromosome, is shown in picture B. The cytoband 13D2 and 19E6 define chromosomal region, which was inverted by FLP/*FRT* site-specific recombination (see Fig. 3-21). Maps are copies from Lindsley and Zimm (1992).

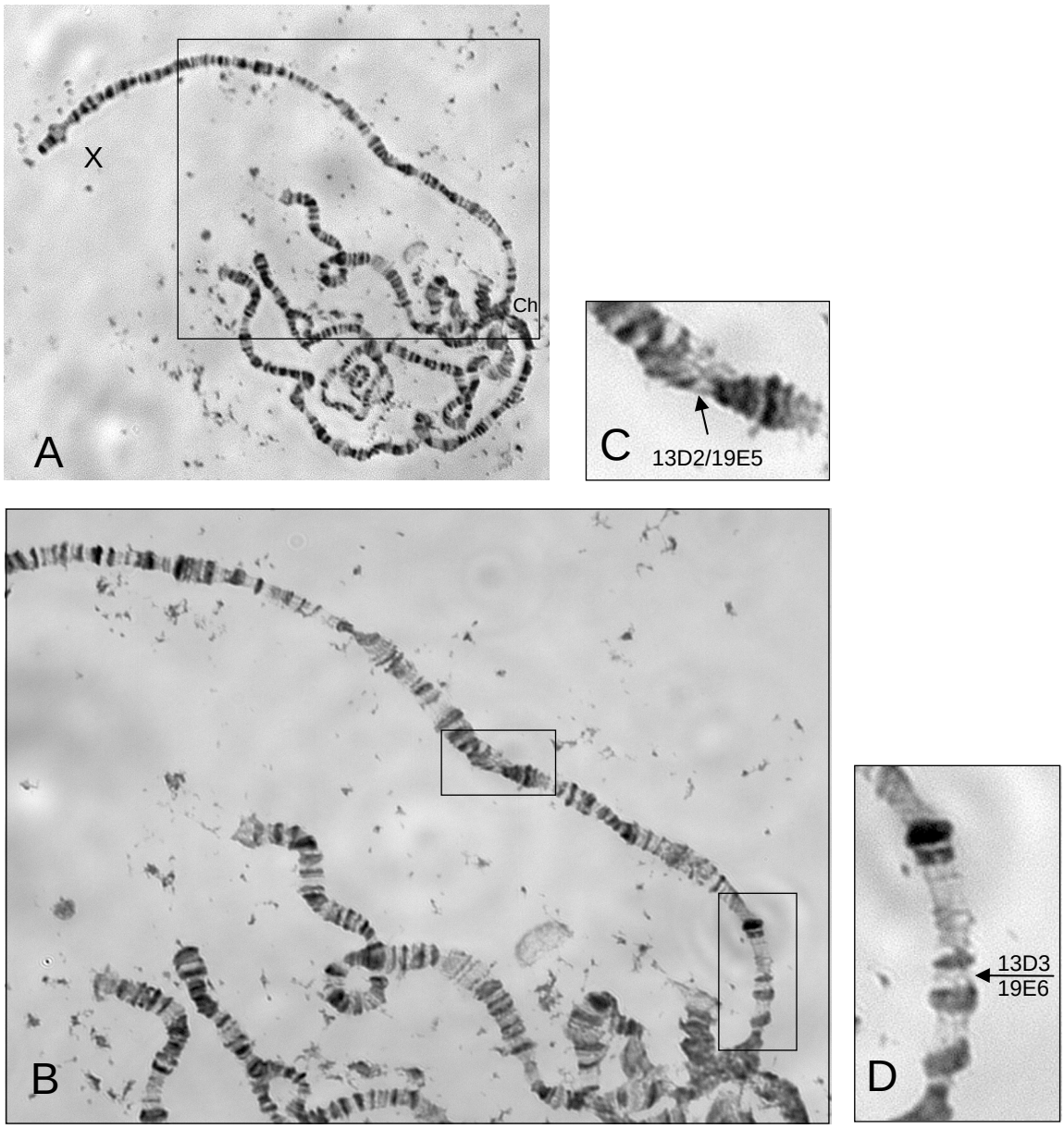


Figure 3-21 Cytology of *X* chromosomal inversion, *In* (*X*) F1/F1.
In picture A, *X* chromosome (X) is the one on the top of other mitotic polytene chromosomes and is connected to the chromocenter (Ch). The magnification of the box (black line) is shown in picture B, where two small boxes define the breakpoints, 13D2/19E5 (picture C) and 13D3/19E6 (picture D), of inverted chromosomal region. This inversion is paracentric, i.e. does not include the centromere. In pictures is shown *In* (*X)* F1/F1 of line #2. Polytene chromosomes were prepared and stained with Giemsa as described in Material and methods, part 2.7.2.

3.3.2 Analysis of defined inversions to test their potential to act as partial balancers

One inversion on the *X* chromosome and five inversions on the third chromosome were analyzed. Information about them, crossing schemes as well as screening principles is described in Material and methods (2.7.1.1 and 2.7.3.1).

There was no recombination observed in the case of the *X*-linked inversion as well as those on the third chromosome (Tab. 3-9). The recombination frequency of 'recombination' control did not correlate with the length of the particular region. In the case of larger

chromosomal regions (14/1 and 26/1), double cross-over caused lower numbers, because flies with such event could not be distinguished from those with parental phenotypes.

Table 3-9 Recombination frequencies on *X* and *III.* chromosomes

	Inversion	Altogether	Rec. frequency (%)	Recombination control	Altogether	Rec. frequency (%)
X chromosome	#1	1,091	0	#1	1,086	21.1
	#2	1,146	0	#2	1,137	18.3
	#3	1,124	0	#3	1,024	20.3
	#4	1,217	0	#4	1,179	16.7
III. chromosome	F2/14	807	0	F2/14	744	4.0
	F2/26	851	0	F2/26	809	9.3
	13/1	825	0	13/1	780	39.0
	14/1	907	0	14/1	954	44.8
	26/1	805	0	26/1	837	43.6

#1, #2, #3 and #4 indicate four independent lines of *X*-linked inversion between *pBac[3xP3-FRT-ECFP]* construct of line F1 and *pBac[PUb-FRT-DsRed]* construct of line F1. On the third chromosome, five different combinations between *pBac[3xP3-FRT-ECFP]* construct (lines 13, 14 and 26) and *pBac[PUb-FRT-DsRed]* construct (line F2 and line 1) were analyzed. Recombination (rec.) frequency was calculated as the number of flies with the recombination phenotype/the number of all analyzed flies. Double cross-over was not distinguishable, because flies with such event should have the same phenotype as their parents.

3.3.3 Re-/immobilization analyses of rearranged *piggyBac* and *Hermes* transposable elements

3.3.3.1 Analyses of rearranged *piggyBac* terminal inverted repeats

In this result part, the question is addressed of how important 5' and 3' terminal inverted repeats (TIRs) of *piggyBac* transposable element (TE) are to its excision/remobilization properties. One inversion on *X* chromosome was analyzed. This contained inverted chromosomal region between *pBac[3xP3-FRT-ECFP]* original *FRT* construct of line F1 and *pBac[PUb-FRT-DsRed]* one of line F1. Four independent lines of this *X*-linked inversion were tested. Rearranged *piggyBac* TE contained either two 3' TIRs (3'TIRs *piggyBac*) or two 5'TIRs (5'TIRs *piggyBac*). Rearrangements of *piggyBac* TIRs, crossing schemes, transposase sources, chromosomal localization of analyzed lines and principles of subsequent screenings were described in Material and methods (part 2.7.1.1 and 2.7.3.2).

Neither the *piggyBac* transposase under the control of *α-tubulin* promoter did cause transposition of 3' TIRs *piggyBac* (Tab. 3-10) nor did transposase under the control of *hsp70* promoter mediate transposition of 5' TIRs *piggyBac* (Tab. 3-12). The *hsp70* promoter driven transposase displayed 100 percent efficiency to excise and newly insert original *pBac[3xP3-FRT-ECFP]* construct (Tab. 3-13) while the *α-tubulin* promoter driven version mediated only 38

percent (the excision rate) and 12 percent (the jumping rate) (Tab. 3-11) of *pBac[PUb-DsRed]* construct. Horn et al. (2003) found that this 'jumpstarter' line with *α-tubulin* promoter driven transposase was very efficient, thus, the resultant low excision and jumping rates rather pointed out the problem of *pBac[PUb-DsRed]* construct. Götschel (2003) already described very low efficiency of the same 'jumpstarter' line to remobilize *pBac[PUb-DsRed]* construct of this *X*-linked line F1. According to obtained data, 3'TIRs *piggyBac* as well as 5'TIRs *piggyBac* can be concluded as efficiently stabilized.

Table 3-10 Re-/immobilization analysis of *pBac3'-3xP3-FRT-DsRed-3'pBac* construct

		Immobilization (m)	Jumping (m)	Excision (f)	Immobilization (f)
Inversion F1/F1	Altogether	no 3xP3-DsRed	3xP3-DsRed	no 3xP3-DsRed	3xP3-DsRed PUb-ECFP
#1	2,246	1,157	0	0	1,089
#2	1,950	998	0	0	952
#3	2,518	1,312	0	0	1,206
#4	2,153	1,122	0	0	1,031
Recombination F1/F1	Altogether	no PUb-DsRed	PUb-DsRed	no PUb-DsRed	3xP3-ECFP PUb-DsRed
#1	2,469	1,266	5	9	1,189
#2	2,335	1,223	2	8	1,102
#3	2,502	1,272	6	9	1,215
#4	2,350	1,214	0	7	1,129

#1,2,3 and #4 are four independent lines of *X*-linked inversion between line F1 of original *pBac[3xP3-FRT-ECFP]* construct and line F1 of *pBac[PUb-FRT-DsRedaf]*. Recombination corresponds to the situation when analyzed chromosomal region is not inverted between these two constructs of F1/F1 lines. To test a remobilization/excision of *pBac3'-3xP3-DsRed-3'pBac* construct, M6.II line of jumpstarter stock containing *Her[3xP3-ECFP;α-tub-piggyBacK10]* construct was used. Immobilization represents flies with such phenotype, which is considered as that one when no excision/remobilization occurs. Jumping means a remobilization event, which is indicated as male (m) flies containing remobilized construct. These flies, however, were not checked for transposase source, so that not all of them represented a new stable insertion. Excision corresponds to female (f) flies, which inherited the chromosome, on which analyzed construct was excised. 3xP3-DsRed = *pBac3'-3xP3-FRT-DsRed-3'pBac* construct; PUb-ECFP = *pBac5'-PUb-FRT-ECFP-5'pBac* construct. PUb-DsRed = the original *pBac[PUb-FRT-DsRed]* construct; 3xP3-ECFP = the original *pBac[3xP3-FRT-ECFP]* construct.

Table 3-11 Jumping and excision rates for re-/immobilization analysis of *pBac3'-3xP3-FRT-DsRed-3'pBac* construct

Transposase source	Recombination	Jumping rate (%)	Excision rate (%)
α-tub-piggyBac	F1/F1	12	38

These results are based on the data of all independent lines of recombination F1/F1 (Tab. 3-10). Jumping rate = the number of vials, which contained at least one male with the original *pBac[PUb-FRT-DsRed]* construct, related to the number of all fertile crosses. In this case, the ration was 8/68. Excision rate = the number of vials, where at least one female without *pBac[PUb-FRT-DsRed]* construct was found, related to the number of all fertile crosses. In this case, the ratio was 26/68.

Table 3-12 Re-/immobilization analysis of *pBac5'-PUb-FRT-ECFP-5'pBac* construct

		Immobilization (m)	Jumping (m)	Excision (f)	Immobilization (f)
Inversion F1/F1	Altogether	no PUb-ECFP	PUb-ECFP	no PUb-ECFP	3xP3-DsRed PUb-ECFP
#1	1,895	941	0	0	954
#2	1,635	817	0	0	818
#3	2,029	986	0	0	1,043
#4	2,028	936	0	0	1,092
Recombination F1/F1	Altogether	no 3xP3-ECFP	3xP3-ECFP	no 3xP3-ECFP	3xP3-ECFP PUb-DsRed
#1	3,126	1,408	93	190	1,435
#2	2,736	1,204	85	167	1,280
#3	2,668	1,210	93	177	1,188
#4	3,110	1,357	100	213	1,440

To test a remobilization/excision of *pBac5'-PUb-ECFP-5'pBac* construct, *piggyBac* transposase source under *hsp70* control of *Mi[3xP3-DsRed; hsp70-piggyBac]_*#1 was used. Definitions are explained in Tab. 3-10.

Table 3-13 Jumping and excision rates for re-/immobilization analysis of *pBac5'-PUb-FRT-ECFP-5'pBac* construct

Transposase source	Recombination	Jumping rate (%)	Excision rate (%)
hsp70-piggyBac	F1/F1	100	100

Definitions are explained in Tab. 3-11. In this case, calculations were done for the original *pBac[3xP3-FRT-ECFP]* construct (Tab. 3-12). In both cases, 100% means that line #1 providing *piggyBac* transposase under *hsp70* promoter mediated the excision or the remobilization of *pBac[3xP3-FRT-ECFP]* construct in 80 vials out of 80 fertile crosses.

3.3.3.2 Analyses of rearranged terminal inverted repeats between *piggyBac* and *Hermes*

Two different inversions between *piggyBac*- and *Hermes*-based constructs on the third chromosome were available in *D. melanogaster*. They contained an inverted chromosomal region between original *FRT* constructs of: (i) line F2 of *pBac[PUb-FRT-DsRed]* construct and line 59 of *Her[3xP3-FRT-EYFP]*, which corresponds to F2/59 inversion; (ii) line 1 of *pBac[PUb-FRT-DsRed]* construct and line 83 of *Her[3xP3-FRT-EYFP]*, corresponding to 1/83 inversion. Each inversion, thus, contained one TIR from *piggyBac* and one TIR from *Hermes* TE. The principle of how *piggyBac* and *Hermes* based constructs were rearranged, their chromosomal localizations, crossing as well as screening schemes with transposase sources were described in Material and methods (part 2.7.1.1. and 2.7.3.3). To determine whether rearranged *piggyBac*/*Hermes* based constructs can still be remobilized in the *Drosophila* genome; two different transposase sources were used: (i) *α-tubulin*-driven *piggyBac* and (ii) *hsp70* promoter-driven *Hermes* transposase.

Although *piggyBac* transposase source under the *α-tubulin* promoter was provided by the same line as was applied in 3'TIRs *piggyBac* analysis, interestingly, the excision rate differed between two original *piggyBac* TE, 43 percent (line F2) and 5 percent (line 1) (Tab. 3-15). The higher rate is very similar to the one that was obtained for line F1 in 3'TIRs *piggyBac* analysis (38 percent). The lower one may reflect potential position effect on line 1. Moreover, there was an estimated null jumping rate for the original *piggyBac* TE. Also in the case of line F1 the jumping rate was three times lower than the excision rate. It suggests that the above mentioned PUb-DsRed1 construct has problems in remobilization. Furthermore, *hsp70* driven *Hermes* transposase also differed in the excision rates, 53 percent for original line 59 and 26 percent for original line 83. Although the difference is not as high as in the case of *α-tubulin* driven *piggyBac* transposase, it points out that also the 83 line suffers from the position effects. Interestingly, the jumping rate based on the strongly expressed 3xP3-EYFP marker was 47 percent for line 59 and 79 percent for line 83. It is surprising that the jumping rate in the case of line 83 was higher than its excision rate. Importantly, the rearranged TEs with one *piggyBac* TIR and one *Hermes* TIR, did not show any excision or new insertion event when *α-tubulin* driven *piggyBac* transposase or *hsp70* driven *Hermes* transposase were provided (Tab. 3-14). The segregation of PUb-EYFP and 3xP3-DsRed should have been observed on condition that either *piggyBac* or *Hermes* transposase can mediate transposition of corresponding TE with one TIR. Nothing like that was found, suggesting immobilization of rearranged *piggyBac*/*Hermes*-based construct.

Table 3-14 Re-/immobilization analysis of rearranged *Hermes*- and *piggyBac*-based constructs

Transposase source	Inversion	Altogether	PUb-EYFP together with 3xP3-DsRed	PUb-EYFP	3xP3-DsRed	no PUb-EYFP no 3xP3-DsRed
				Remob/ Exc	Remob/ Exc	
Scored in			Sb^+	TM3 Sb/ Sb^+	TM3 Sb/ Sb^+	TM3 Sb
α-tub-piggyBac	F2/59	1,723	956	0/0	0/0	767
	1/83	1,726	910	0/0	0/0	816
hsp70-Hermes	F2/59	1,012	525	0/0	0/0	487
	1/83	2,325	1,125	0/0	0/0	1,200
Transposase source	**Recombination**	**Altogether**	**PUb-DsRed together with 3xP3-EYFP**	**PUb-DsRed**	**3xP3-EYFP**	**no PUb-DsRed no 3xP3-EYFP**
				Remobilization	Excision	
Scored in			Sb^+	TM3 Sb	Sb^+	TM3 Sb
α-tub-piggyBac	F2/59	2,434	1,427	0	9	998
	1/83	2,307	1,339	0	2	966
				Excision	Remobilization	
Scored in			Sb^+	Sb^+	TM3 Sb	TM3 Sb
hsp70-Hermes	F2/59	1,828	997	11	9	811
	1/83	2,602	1,370	6	29	1,197

PUb-EYFP = *pBac[PUb-FRT-EYFP]Her* construct; 3xP3-DsRed = *Her[3xP3-FRT-DsRed]pBac* construct; PUb-DsRed = the original *pBac[PUb-FRT-DsRed]* construct; 3xP3-EYFP = the original *Her[3xP3-FRT-EYFP]* construct. F2/59 and 1/83 represent two different combinations of *FRT* lines on the third chromosome. *FRT* lines F1 and 1 contained the original *pBac[PUb-FRT-DsRed]* construct; *FRT* lines 59 and 83 contained the original *Her[3xP3-FRT-EYFP]* construct. PUb-EYFP in TM3 Sb background indicates a remobilization (Remob) of *pBac[PUb-FRT-EYFP]Her* construct and in Sb^+ background indicates an excision (Exc) of *Her[3xP3-FRT-DsRed]pBac* construct. 3xP3-DsRed = in TM3 Sb background indicates a remobilization (Remob) of *Her[3xP3-FRT-DsRed]pBac* construct and in Sb^+ means an excision (Exc) of *pBac[PUb-FRT-EYFP]Her* construct. TM3 Sb = balancer chromosome.

Table 3-15 Jumping and excision rates

Transposase source	Recombination	Jumping rate (%)	Excision rate (%)
α-tub-piggyBac	F2/59	0	42.9 (6/14)
	1/83	0	5 (1/20)
hsp70-Hermes	F2/59	46.6 (7/15)	53.3 (8/15)
	1/83	79 (15/19)	26.3 (5/19)

Jumping and excision rates are calculated as the number of vials, in which at least on fly with searched construct was found and this value was related to all fertile crosses. The ratio is shown in parentheses (the number of vials/the number of all fertile crosses). Both rates are based on the data of F2/59 or 1/83 recombination (Tab. 3-14). Jumping rate (*α-tub-piggyBac*) = a remobilization of the original *pBac[PUb-FRT-DsRed]* construct; jumping rate (*hsp70-Hermes*) = a remobilization of the original *Her[3xP3-FRT-EYFP]* construct. Excision rate (*α-tub-piggyBac*) = the excision of the original *pBac[PUb-FRT-DsRed]* construct; excision rate (*hsp70-Hermes*) = the excision of the original *Her[3xP3-FRT-EYFP]* construct.

3.3.4 Establishment of duplications and deletions by site-specific recombination on homologous chromosomes

Two different combinations of *FRT* lines were analyzed: (i) line M2 of *pBac[Pub-FRT-DsRed]* construct and line 34 of *pBac[3xP3-FRT-ECFP]* one, (ii) line 24 of *Her[3xP3-FRT-EYFP]* construct and line F2 of *pBac[Pub-FRT-DsRed]*. Both combinations were on the third chromosome (3L). The principle of FLP/*FRT* site-specific recombination in *trans*, i.e. on homologous chromosomes, chromosomal localization of *FRT* lines, crossing schemes as well as the screening strategy was described in Material and methods (part 2.7.1.2 and 2.7.3.4).

To drive a flipase source in male germ-line cells, temperature sensitive *hsp70* promoter (DrosDel Kit, www.drosdel.org.uk) was used. *hsp70* promoter that is inducible in male germ-line cells except of primary spermatocytes (Bonner et al. 1984) was efficient to drive the flipase source, which mediated FLP/*FRT* recombination, resulting in duplications and deletions only in the case of 24/F2 combination (Tab. 3-16). Surprisingly, half out of all 24/F2 crosses set ups were sterile (20 out of 44). There were almost one third of fertile crosses (8 out of 22), which contained flies with either a duplication or a deletion. Duplications and deletions were found in roughly 1.8 percent of all analyzed flies (Tab. 3-16).

Table 3-16 Establishment of duplications and deletions in *trans* position

***FRT* line combination**	**Distance between *FRT* sites (~kb)**	**Duplication**	**Deletion**	**Altogether**
M2/34	160	0	0	6,194
24/F2	56	31 (1.26%)	13 (0.53%)	2,468

The percentage of duplications and deletions = the number of flies with the duplication or the deletion related to all analyzed flies for the particular *FRT* line combination. Lines M2 and F2 = the original *pBac[Pub-FRT-DsRed]* construct; line 24 = the original *Her[3xP3-FRT-EYFP]* construct; line 34 = the original *pBac[3xP3-FRT-ECFP]* construct. Duplications were found in 8 out of 44 set ups (18.2%). Deletions were found in 5 out of 44 set ups (11.4%).

Flies with the duplication were more frequently found than those with the deletion (31 vs. 13, respectively). A ratio between females and males with the duplication was 12:19 while with the deletion 8:5. A segregation distortion was statistically non-significant for deletions as well as duplications ($P>0.05$). Interestingly, flies with the duplication were accompanied by those with the deletion in 5 out of 8 crosses. There were only flies with the duplication in the remaining 3 out of 8 crosses. On the other hand, flies with the deletion were never found alone and were always accompanied by those with the duplication. Original *FRT* lines and final lines with either the duplication or the deletion event are shown in Fig. 3-22 and Fig. 3-23, respectively.

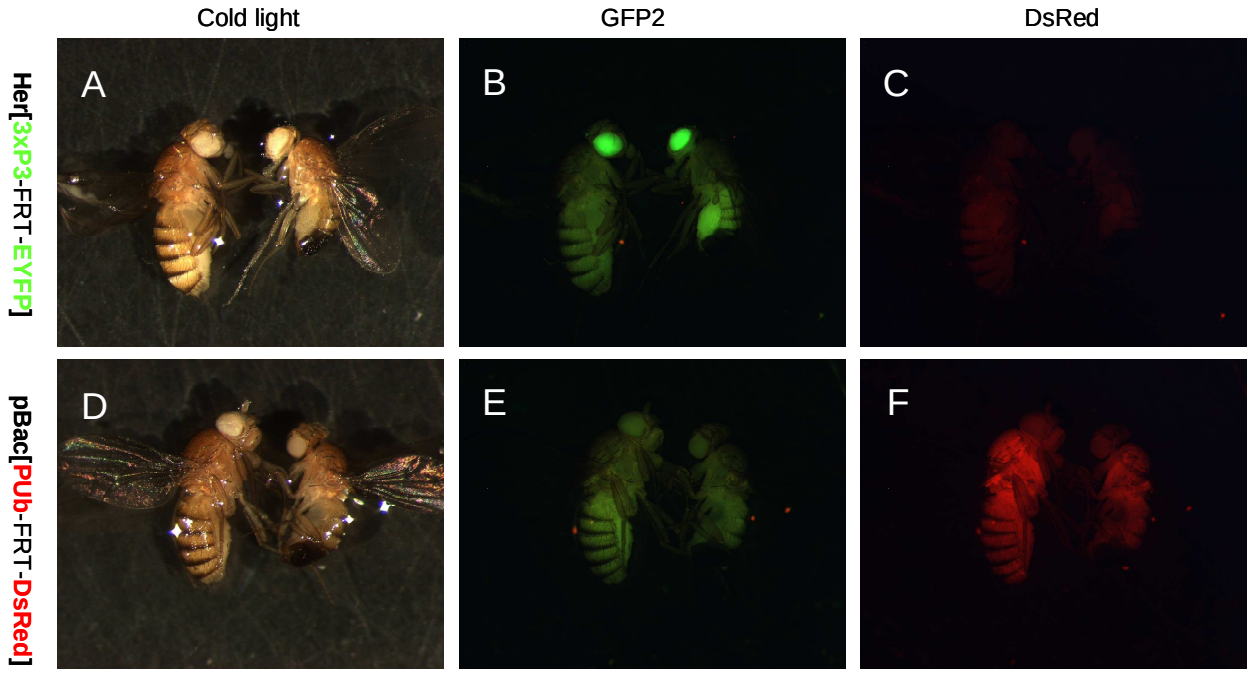


Figure 3-22 Original lines.
Visible 3xP3-EYFP is shown in picture B for *Her[3xP3-FRT-EYFP*] construct (A, B, C). DsRed1 marker under PUb promoter is obvious in picture F for *pBac[PUb-FRT-DsRed]* construct (D, E, F). Note that images taken under GFP2 (B, E) and DsRed filter (C, F) are with the same exposition time (30 s) to show as to how distinguish fluorescent markers under same conditions when flies were screened. Left is female, right is male fly.

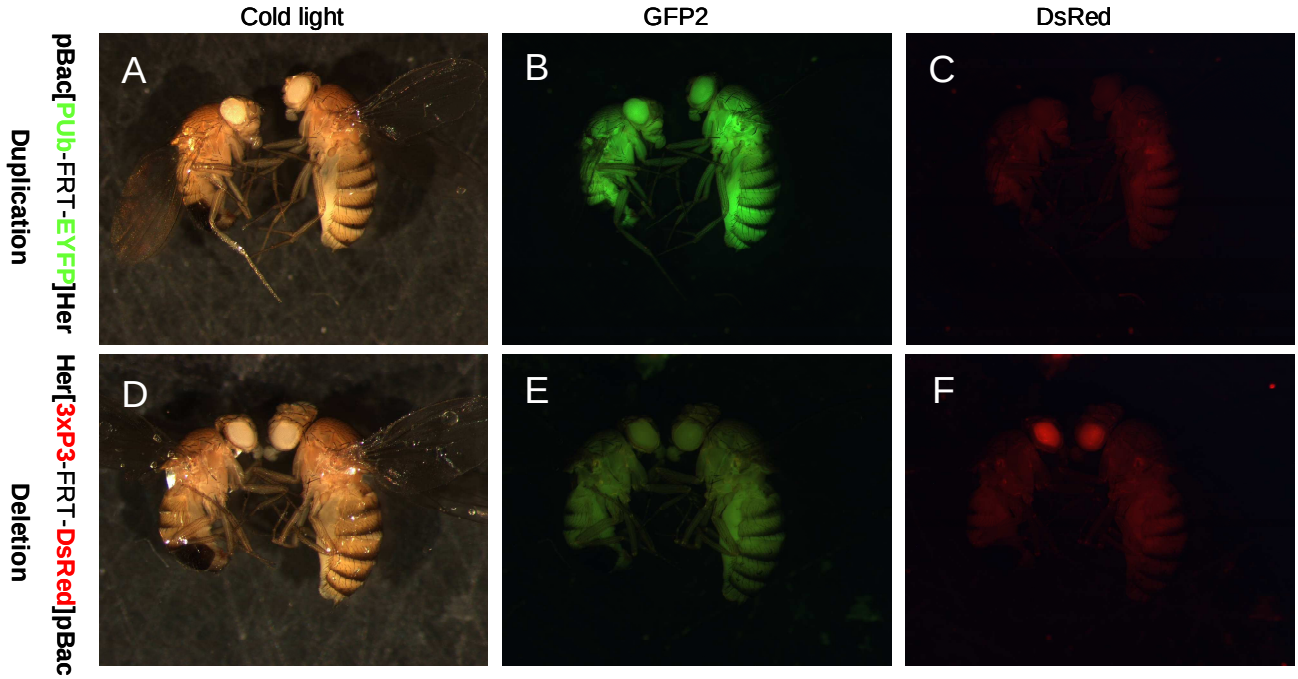


Figure 3-23 Duplication and deletion created by FLP/*FRT* recombination.
Strong ubiquitous PUb-EYFP expression was found corresponding to duplication (B). On the other hand, deletions were identified according to a weak 3xP3-DsRed1 expression (F). By this act of FLP/*FRT* recombination, terminal parts of original *piggyBac* and *Hermes* based constructs were combined (left site next to images. A, B, C represent flies with duplication while D, E, F show flies with deletion. Pictures (B, C, E, F) were taken under the same conditions (30 s) to compare intensity of markers. Left is male, right is female fly.

4 DISCUSSION

4.1 TESTING OF *DROSOPHILA* HEAT-SHOCK PROMOTER IN THE RED FLOUR BEETLE, *T. CASTANEUM*

When transgenic larval, pupal and adult stages of *Tribolium castaneum*, which contained EGFP marker under the control of *Drosophila hsp70* promoter (*Dmhsp70* promoter) in *piggyBac* backbone, were exposed to the increased temperature of 47℃ in the incubator for 1h, they displayed induced EGFP expression nearly in the whole body parts. It is in agreement with observation in *D. melanogaster*. The *Drosophila hsp70* promoter is widely used there for its feasibility to transiently drive transgene expression almost in all cells or tissues at any time during fly development (Halfon et al. 1997), on condition that these cells are exposed to the increased temperatures. It reflexes the heat-shock inducible manner of broadly conserved thermotolerance factor Hsp70. The Hsp70 proteins are undetectable in *Drosophila* cells at normal temperatures, but become abundant when they are exposed to high temperatures (Valazquez et al. 1983). Its rapid and massive induction is one of the highest among metazoan proteins (Wang and Lindquist 1998). In sum, the *hsp70* promoter isolated from *D. melanogaster* was proven to be functional and can be transiently activated in the red flour beetle, *T. castaneum*. The promoter functionality in evolutionarily distant species, *D*. *melanogaster* and *T*. *castaneum*, provides the proof about the high conserved character of this promoter.

Dmhsp70 promoter inducibility, *in vivo*, suggested a possibility for using this promoter in *piggyBac*-based mutagenesis screen in *T. castaneum*. However, a low constitutive EGFP expression was observed and varied in transgenic lines of this species. In *D. melanogaster*, it is known that *hsp70* genes maintain an open chromatin configuration at normal temperatures (Wu 1980) and basal level of transcription from the heat-shock promoter can be observed in this species (Yost et al. 1990). In regard to transposon-based mutagenesis screen, such behaviour has to be taken into account. It was shown that *Tribolium* line, containing *piggyBac* source under the *Dmhsp70* promoter in *Minos* construct, mediated 85 percent of new transposition events without heat-shock application (S. Brown, personal comm.). Therefore, this promoter provides rather constitutive expression of transposase source at least in some transgenic lines. The question is whether a transient induction can be mediated in the *Tribolium* germline. This might depend on the particular line, and lines like line A with the highest inducibility, should be tested one more time in detail. Although florescent marker expression driven by *Dmhsp70* promoter was observed in ovaries and testes, it was impossible to clearly state whether this happened in soma- or germ-line cells. Firstly, one could polemize whether the response of *Dmhsp70* promoter corresponds to the exact place of its induction due to time lag between the heat-shock treatment and the dissection of reproductive organs. And secondly, distinct morphology of *Tribolium* reproductive organs from those of *D. melanogaster* made it difficult to

clearly identify the place of somatic or germ-line cells, which necessitates further microscopic analyses.

As no line with very low basal activity of *Dmhsp70* promoter might be available, other alternative promoters such as promoters directly isolated from *T. castaneum* will be worth trying. The promising might be *Tribolium hsp70* promoter (recently under way in E. A. Wimmer lab, personal comm.). However, this will require further tests in this species, prior to its usage there.

All requirements are set up for carrying out a transposon-based insertional mutagenesis screen in *Tribolium castaneum* to identify interesting mutant phenotypes of its odoriferous glands. This is especially of an importance as biological functions of these glands can be asked and such information is not available in *D. melanogaster.* Several observations showed that *Tribolium* odoriferous (also called “defensive” or “stink”) glands (Fig. 4-1) produce excretions, which accumulate in flour medium and in dense populations may subsequently wipe out all specimen (Sokoloff 1972). The reason is the accumulation of quinones and other volatile substances in survival means. Interestingly, their high concentrations consequently may affect critical periods in the development of larvae or pupae, resulting in various non-heritable abnormalities. Thus, the knowledge about such processes on genetic level will inevitably contribute to the understanding of the communication between beetles and their behavioural biology.

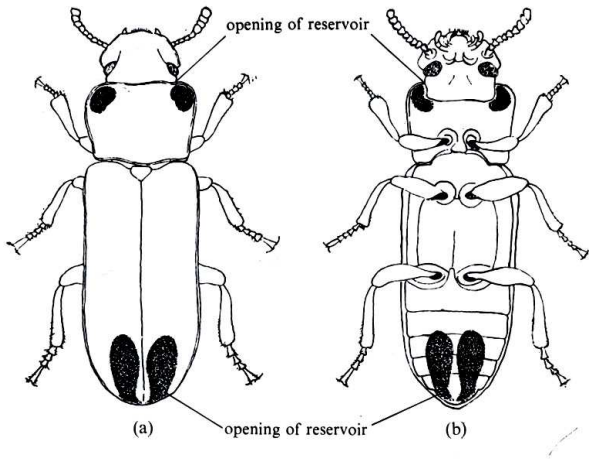


Figure 4-1 Odoriferous glands in *Tribolium castaneum*.
There are two pairs of odoriferous glands in all *Tribolium* species (Sokoloff 1972); one pair is located in the thoracic and the other in abdominal cavity. They can be identified as colourless reservoir structures, producing yellowish, oily, highly volatile fluid. The colour differs during beetle aging, becoming purplish-black in old beetles. A similar appearance was found in the mutant *melanotic stink glands*, *msg*, producing excretions of dark crystalline mass, which was identified also in another mutation, *tar*. (Hoy and Sokoloff 1965). (a) dorsal view, (b) ventral view.

Another question is whether all cells in *T. castaneum* respond to the heat-shock. In *Drosophila*, for example, Hsp70 cannot be induced in preblastoderm embryo (Graziosi et al. 1980). Similar embryonic restrictions of Hsp70 expression exist in other invertebrate and vertebrate organisms, which is presumably due to detrimental effects of Hsp70 on cell division during early embryogenesis (Wang and Lindquist 1998).

Lastly, transgenic animals might be used in pest management programs in the future. Horn et al. (2003) showed that sterile insect technique (SIT) may be improved by using the embryo-specific lethal system, which requires spatially and tissue-specific promoters. This system is based on broad-range promoters, which are functional also in other non-dipteran species. Thus, reliable promoters will be required in those. In regard to *Dmhsp70* promoter, however, its basal activity and temperature sensitivity are not suitable for pest management programs.

4.2 DEVELOPMENT OF ASSAY SYSTEMS FOR THE EVALUATION OF BINARY EXPRESSION SYSTEMS

4.2.1 Comparison of binary expression systems in *Drosophila melanogaster*

Misexpression studies provide valuable information about cell division, cell- and tissue development and other relevant biological functions. For this purpose, binary expression systems are used. In this thesis, the yeast Gal4/*UAS* system (Brand and Perrimon 1993, Rørth 1998) was compared with two bacterial systems: LexA/$(LL)_4$ (Szüts and Bienz, 2000) and the tetracycline-controlled one (Bello et al. 1998, Krueger et al. 2003). Although these systems are widely used in the model organism, *D. melanogaster*, no comparison of their working efficiencies has been available so far, certainly simplifying the choice of a suitable one for a particular experiment in this species.

The comparative analyses of these systems based on the measurement of the reporter kinetics revealed that the eukaryotic yeast Gal4/*UAS* system is the best working one while the bacterial systems with DNA-binding domains of repressor proteins LexA and TetR, respectively, performed considerably lower transcriptional potential in eukaryotic cells of the fruitfly, *D. melanogaster*. This likely suggests the differences between the bacterial and the eukaryotic transcriptional machinery and their influence on the working efficiencies of these systems.

In the budding yeast, *Saccharomyces cerevisiae*, Gal4 transcriptional regulatory protein binds to four sites of the GAL upstream activating sequence (*UASG)* of the adjacent GAL1 and GAL10 genes under induced conditions, i.e. by a presence of the carbon source, galactose, resulting in GAL genes transcription (Giniger et al. 1985). Fischer et al. (1988) showed that such system can be used in *D. melanogaster* for a tissue-specific transcription when *hsp70 Drosophila* basal promoter was placed under *UAS* binding sites (*UAST*). Based on that, Brand

and Perrimon (1993) develop a genetic tool for the targeted gene expression in somatic tissues of *D. melanogaster*. And moreover, Rørth (1998) contributed by overcoming the soma limitations of *UAST* by its adjustment to germ-line tissues (*UASp*) in *D. melanogaster*. In parallel, detailed deletion analyses of Gal4 transcriptional potential in the yeast showed that main part of Gal4 protein can be deleted without loosing its stimulation potential for the transcription of *UAS*-linked genes and only Gal4 minimal domains, N-terminal DNA-binding and C-terminal transcriptional activating one, are required (Ma and Ptashne 1987). The establishment of various Gal4 chimeric regulatory proteins led to increase/decrease of its transcriptional potential. Brent and Ptashne (1985) found that Gal4 DNA-binding domain can be replaced by prokaryotic one of LexA repressor and still maintain the transcriptional activity in the yeast. Similarly, Sadowski et al. (1988) replaced Gal4 activation domain by VP16 one of *Herpes simplex* virus, resulting in unusual transcriptional efficiency in mammalian cells.

In this thesis, original Gal4, chimeric Gal4VP16 and a deletion version, Gal4Δ, were analyzed for their transcriptional potential when binding either *UAST* or *UASP* responder. Interestingly, original Gal4 that is predominantly used for misexpression studies performed the lowest potential to stimulate the reporter gene, *lacZ*, transcription in photoreceptors of *D. melanogaster* in comparison with the moderate potential of the chimeric Gal4VP16 and the highest one of its deletion version, Gal4Δ. The higher potential of the chimeric Gal4VP16 fulfilled expectations based on previous results of Sadowski et al. (1988) in mammalian cells. However, one has to take into account that its moderate potential is based on one active Gal4VP16 line in this thesis. Thus, more lines will be required to state whether the potential of this chimeric version is closer to the original Gal4 or its deletion version, Gal4Δ. The reason why only one line could have been analyzed emerged from the difficulties to get more active lines of Gal4VP16, suggesting its toxic effect on host cells of *D. melanogaster*. It is not as surprising, because already Driever et al. (1989) referred about a deleterious effect on the anterior development of *Drosophila* embryos by using BcdVP16 fusion protein even when its mRNA was in low concentrations. Other experimental observations have also suggested the difficulties to set up Gal4VP16 lines in *D. melanogaster* (Ch. Lehner, personal comm.). On the other hand, however, when such lines are obtained, they display more potent transcriptional activation than the original Gal4 regulatory protein. It suggests that the potential of other new lines of the chimeric Gal4VP16, which will be established for further experiments, are expected to perform a higher potential than the original Gal4. And moreover, the line of the chimeric Gal4VP16, which was analyzed in this thesis, was likely the weakest and the less toxic one. Furthermore, surprisingly the deletion version, Gal4Δ, stimulated twice effectively the reporter activation than the original Gal4 and showed the highest transcriptional potential of all analyzed versions. It fulfilled an expectation that this version should be more stable and least toxic in *Drosophila* cells than its original Gal4 version (G. Struhl, personal comm.). In contrast, Ma and Ptashne (1987)

demonstrated that overexpression of this deletion version, Gal4Δ, was twice less potent to stimulate the reporter gene, *lacZ*, activation than the Gal4 original one in yeast cultures. The recent discoveries that transcriptional activator proteins are regulated by proteasome-mediated degradation showed that the rate of activators degradation correlates with activation domain potency *in vivo* (Molinari et al. 1999). Firstly, it explains differences between the original Gal4 and the chimeric Gal4VP16 containing the potent activation domain of *Herpes simplex* virus. And secondly, it may suggest that Gal4Δ degradation is faster than those of other versions in *D*. *melanogaster* unlike in yeast. The efficient recruitment of activator-target protein complexes to the promoter means that they are subject to rapid degradation of proteasome (Molinari et al. 1999). Such degradation of Gal4 regulatory protein is mediated by F-box protein Dsg1/Mdm30 of Skip-Cullin-F-Box (SCF) complex by ubiquitin-like proteolysis in yeast (Muratani et al. 2005). As the yeast as well as all cells of *D*. *melanogaster* possess the eukaryotic transcriptional machinery, it may point out, rather than a different efficiency in Gal4Δ-protein complex assembly to the promoter in these eukaryotic organisms, the distinct types of proteolytic machinery. In *D*. *melanogaster*, the cell-cycle analyses revealed the well-characterised anaphase-promoting complex (APC) that is required for metaphase-anaphase transition. Thus, it raises an interesting question whether transcriptional activators are also degraded by proteins of this evolutionarily conserved complex or by other ones. Recently, the conserved F-box protein related to Cdc4p, Slimb, was proposed to participate in growth and modifying Hedgehog (Hh) and Wnt/Wingless (Wg) pathways, mediating the processing and the degradation of Hh and Wg target transcriptional factors, Cubitus interruptus and Armadillo by ubiquitin proteolysis (Jiang and Struhl 1998). The presence of other SCF homologs such as dCullin1 or SkpA, which were likely identified in *D*. *melanogaster* by Bocca et al. (2001), and the evidence of the conserved character of SCF pathways in plants, animals or viruses (Patton et al. 1998) suggest that the misexpression of the yeast Gal4 regulatory protein in the eukaryotic photoreceptor cells of *D*. *melanogaster*, lacking Gal4 endogenous basal expression unlike in yeast, may be regulated by proteins of both, APC or SCF complex, or potentially also other Ub-like ones (e.g. NEDD8).

In this thesis, the data also suggested that there is the dependence on the character of flanking sequences of binding sites in *UAST* and *UASp*. In all cases of Gal4 versions, their transcriptional activation capability is higher in combination with *UAST* responder than with *UASp* one in eye tissue of *D*. *melanogaster*. The Gal4 variants in combination with *UASp* displayed values that are about one third of the values of Gal4 variants in combination with *UAST*. The *UASp* responder is designed to be active also in the germline throughout female oogenesis (Rørth, 1998), which probably affects its potential in somatic tissues. Moreover, these results also showed that the ratio between Gal4 variants/*UAST* and Gal4 variants/*UASp* is maintained. In regard to the discussed yeast and *Drosophila* contradiction in Gal4 and Gal4Δ

working efficiency, it points out that the difference between these two regulatory proteins to stimulate the reporter transcription in yeast and the eyes of *D. melanogaster* has not been likely caused by their distinct potential to bind the original *UASG* in yeast and *UAST* or *UASp* in *Drosophila* eyes. Ma and Ptashne (1987) showed that the values, which they obtained for Gal4 mutants carried on plasmids, were not overestimates of the Gal4 intrinsic activity. They stably integrated a few mutants into chromosomes and found that values were not lowered. Moreover, they also mentioned that higher levels of Gal4 can not result in more Gal4 molecules bound to the *UASG*. However, their experiments cannot exclude the low level of the wild-type Gal4 protein that, as they surprisingly suggested, is sufficient to fill the binding sites of the *UASG*. Although their results, thus, could have been influenced by the competition between the wild-type Gal4 and Gal4 mutants, resulting in lower transcriptional potential, it is inconsistent with their other observations that remained unexplained. They found that amino-terminal with 196 and 238 residues still activate transcription, however, other longer amino-terminal Gal4 mutants, including these residues together with the following residues from the carboxyl terminus, lost their transcriptional potential, although they bound DNA. Therefore, the lower values that were observed for Gal4Δ should not have been caused by a competition between this deletion variant and the low levels of the wild-type Gal4 original protein in their binding the *UASG*. It rather suggests its altered accessibility to be efficiently degraded due to the modification of their protein assembly. Although it is a pure speculation, it would be an interesting task to re-check experiments done by Ma and Ptashne (1987) and evaluate the amount of transcriptionally active Gal4Δ mutants in yeast as well as in *D*. *melanogaster*. It should be possible as shown by Muratani and co-workers (2005), who have developed a method to distinguish the pool of Gal4 proteins, which are activating or has activated the reporter transcription. It is based on the fact that Gal4 regulatory protein is phosphorylated by RNA polymerase II as a consequence of Gal4 interaction with the transcriptional machinery. The active form of Gal4, isoform ‘c’ (Gal4c), is distinguishable from those non-phosphorylated and inactive, ‘a’ and ‘b’. The authors have revealed that the active Gal4c is most notably phosphorylated on serine residues, S699 and S837, besides other ones. Active Gal4Δ deletion variant that was analyzed in this thesis possesses only S837 while all other non-active amino-terminal Gal4 deletion mutants with amino-terminal residues that were analyzed by Ma and Ptashne (1987) are missing both, S699 and S837. Anyway, other phosphorylation sites should result in a potential recognition of the active Gal4c, which undergoes rapid degradation under inducing conditions in yeast, i.e. in galactose, while Gal4a/b are stable. Curiously, the overexpression analyses of Gal4 deletion mutants under *ADH1* promoter (Ma and Ptashne 1987) were carried out in yeast cultures with the carbon source, i.e. in inducing conditions that is important for the activation of *dsg1* gene and thus also for the efficient Gal4c ubiquitin-mediated proteolysis. In *D*. *melanogaster*, however, expected Gal4c rapid degradation of Gal4Δ will be probably initiated by other

induced/non-induced processes than the presence/absence of the carbon source, suggesting other ubiquitin-like degradation machinery, which is rather controlling the presence of non-host proteins in the cell. Based on that, the comparative study of Gal4Δ variants in yeast and other eukaryotic species, *D*. *melanogaster*, may shed a light on the ubiquitin-like degradation processes of transcriptional activators.

The observation that the bacterial LexA/*(LL)*$_4$ chimeric system is not as efficient as the Gal4/*UAS* one in photoreceptors of *D*. *melanogaster*, may suggest differences in the bacterial vs. eukaryotic machinery. It also showed that LexA, DNA-binding domain from *Escherichia coli* fused to the yeast Gal4 activation domain, is functional in adult eye tissue of *D*. *melanogaster*. This system was previously tested only in embryonic tissues (Szüts and Bienz, 2000).

Finally, the bacterial tetracycline-controlled system (Gossen and Bujard 1992) represents an advanced version of binary expression systems compared to Gal4/*UAS* and LexA/*(LL)*$_4$. It can be conditionally regulated by the presence/absence of the antibiotic, tetracycline or doxycyline, and consists of the tetracycline repressor protein, TetR, which is fused to the potential VP16 activation domain of *Herpes simplex* virus. Bello et al. (1998) has introduced this version, which necessitates no antibiotic to be functional, into *D*. *melanogaster*. Importantly, the fusion protein, tTA, activates a gene transcription from *TRE* only on condition that its dimer is assembled, which may influence its transcriptional potential in host cell environment. Thus, Krueger et al. (2003) converted this dimeric allosterical regulatory protein, tTA, into a fully functional monomer, sctTA, that is connected by a 29 amino acid linker. This alleviates the dimerisation process of tTAs in host cells. In this thesis, tTA, sctTA and reverted version of sctTA, which requires tetracycline to be functional, scrtTA, were tested. None of these displayed efficient transcriptional potential in comparison to Gal4/*UAS* and LexA/*(LL)*$_4$ systems. Although the sequence of all variants was confirmed and 3xP3 driven sctTA as well as scrTA proteins were present in head samples, they were not able to stimulate efficient reporter gene activation by binding functional *TREs*. It is disputable whether this bacterial system is so weak in comparison to Gal4/*UAS* and LexA/*(LL)*$_4$. However, it is consistent with rare enhancer detection by using tTA/*TRE* system in contrast to Gal4Δ/*UAS* in an insertional mutagenesis screen (Horn et al. 2003). The authors already suggested that the low sensitivity of this system is likely due to a lack of effective expression amplification of this binary system, which supports the obtained data in this thesis. Lastly, the single chain version, sctTA, was expected to possess higher transcriptional potential (Ch. Berens, personal comm.), but this was not observed. On the other hand, the high levels of the protein presence of 3xP3-sctTA and undetectable protein level of 3xP3-tTA suggests that the transcriptional potential of the transactivator does not necessarily correlate with its protein level. Stebbins et al. (2001) showed that undetectable tTA protein under the *actine5C* promoter mediated an efficient and twice higher production of the reporter protein, luciferase, than its reverted altered version *actin5C*

driven rtTA-M2, which was detectable in extracts from *D. melanogaster*. Taken this together with similar observations in HeLa cells (Knott et al. 2002), reverted versions are less potent than their non-reverted homologs. Thus, it is not surprising that scrtTA protein, although present, did not stimulate any reporter production.

In sum, a kinetic measurement of the reporter protein, β-galactosidase, was developed and used for the evaluation of three different binary expression systems in photoreceptors of *D. melanogaster*. The transcriptional potential has decreasing tendency from the yeast Gal4/*UAS* towards the bacterial LexA/*(LL)*$_4$ and the tetracycline one in this species. These results provide the information about their working efficiency in this species and contribute to decision which of these systems will be the most suitable for a particular experiment. Curiously, they also pointed out potential differences between the degradation processes of transcriptional regulatory proteins in yeast and *D. melanogaster*.

4.2.2 Assay systems for evaluation of Gal4/*UAS* system variants in *Tribolium castaneum*

Gal4Δ/*UAST* and Gal4VP16/*UAST* systems were preliminarily tested in *Tribolium castaneum*. Although these systems displayed the highest working efficiencies in *D. melanogaster*, there were negative results obtained in *T. castaneum* by using: (i) X-gal assay, (ii) β-gal kinetics and (iii) direct tests for the protein presence of the β-gal reporter. None of analyzed transactivators showed a potential to induce the reporter protein. Unlike in adults of *D. melanogaster*, surprisingly high background (the endogenous ß-gal activity) was observed in larval (not shown) and pupal tissues of *Tribolium* by using X-gal assays. This suggests that X-gal assay is not suitable for *T. castaneum.* Moreover, the weakness of this system was observed in transgenic zebrafish (Scheer and Campos-Ortega 1999), transgenic *Xenopus* (Hartley et al. 2002) as well as in transgenic *Bombyx mori* (Imamura et al. 2003). This suggests that analyzed system variants might also be very weak in *T. castaneum* and potential silencing/inhibition of Gal4Δ and Gal4VP16 could happen. Taken it all together, negative results could have been caused by several reasons; the β-gal degradation, its high endogenous activity or very low amount of this protein in analyzed extracts due to the weakness of analyzed system variants, ‘dotted’ pattern in the eyes as well as insufficient amount of heads per extract.

In sum, to figure out whether or not Gal4/*UAS* functions in *T. castaneum* the following may help: (1) other reporter gene (luciferase or enhanced fluorescent protein); (2) toxic reporter gene, so that even low amounts will be detectable in early embryo stage or later when Gal4/*UAS* system is combined with transiently inducible promoter; (3) tests of original Gal4/*UAST* system (or adjustment of *UASG* for *T. castaneum*) or (4) to drive this system by using *Tribolium* endogenous promoters and (5) as already discussed in *D. melanogaster* part

4.2.1, if possible, further tests for the presence of functional mRNA with a focus on a degradation/stabilization of the active and the inactive isoform of Gal4 regulatory protein in this species.

4.3 NON-P-ELEMENT BASED CHROMOSOMAL REARRANGEMENTS BY USING FLP/*FRT*-MEDIATED RECOMBINATION IN *DROSOPHILA MELANOGASTER*

4.3.1 Balancer chromosomes

Designed inverted chromosomal regions on *X* and the third chromosome have caused suppression of meiotic recombination in all tested cases in the model organism *D. melanogaster*. These inversions were created by technique (Götschel 2003), which is based on broad-range transposable elements, *piggyBac* and *Hermes*, making it feasible for introduction into *T. castaneum*. Universal eye-specific 3xP3 promoter and constitute *polyubiquitin* one were used. The first one was proved to be functional in *T. castaneum* (Berghammer et a. 1999) and the latter has recently been isolated from this species (Lorenzen et al. 2002), thus, such technique is highly expected to be functional there. Moreover, EYFP and DsRed1 fluorescent markers work in this beetle and their usage facilitate easy identification of these inversions, i.e. in generating partial balancers. The easy identification based on fluorescent markers will enable to recognize balanced stocks and obviate laborious sorting of non-transgenic beetles every generation according to their transformation markers. However, balancer homozygots will have to be sorted out as well to avoid from a mutation loss. In *T. castaneum*, their manual selection will be time consuming and can be obviated by introduction of recessive lethal mutation/s onto partial balancer chromosomes.

Prior to the introduction of the novel technique (Götschel 2003), the other question must be determined whether the yeast FLP recombinase can act on *FRT* target sites to mediate the site-specific recombination in *T. castaneum* (recently tested in M. Klingler lab, personal comm.). If this is functional, it will provide an advantageous approach for *T. castaneum* genetics. Defined inversions could be established in the chromosomal region of obtained mutations and balance these mutations in *T. castaneum*. This enables to maintain various mutant phenotypes, which will be obtained from the transposon-mutagenesis screens, accelerate identification of these mutations or identify homozygous embryonic-lethal ones. It beneficially contributes to overcome the limitations to only a few balancers (Beemann et al. 1986), covering a small part of *Tribolium* genome that is in future insufficient for mutations obtained by transposon-based mutagenesis screens.

4.3.2 Stabilization of broad-range transposable elements

Genomes are principally stable, preserving structural information about nucleic acids and proteins for next generations. To their modifications contribute the accumulation of several mutations, meiotic recombination and also transposable elements (TEs). Those latter possess a function enabling them autonomously mobilize in the genome or even among genomes. There are two classes of TEs. The most important class in regard to insect transgenesis is class II, whose TEs transpose directly from DNA to DNA, have mainly short terminal inverted repeats (TIRs) and contain its own transposase source. However, only a little is known about their origin. TEs usually belong to a TE family, which could originate in one species or is a consequential result of lateral and horizontal transmission from another species (Dröge et al. 1998, Hoy 2003).

In *D*. *melanogaster*, TEs of both classes were identified, e.g. long interspersed repeats (LINE) of Class I and importantly e.g. *P* elements, *hobo*, *mariner* or foldback (FB) elements of Class II (reviewed in Hoy 2003). They can excise, insert or cause chromosome rearrangements such as inversions, deletions or translocations, thus, contributing to genome evolution.

Insect transgenesis and the germ-line transformation of *D. melanogaster* (Rubin and Spradling 1982, Spradling and Rubin 1982) are based on non-autonomous *P* elements and transient transposase source. The ability to stably integrate *P* elements was provided by deletions of its own transposase and introduction of such element to *Drosophila* strain that is lacking *P* transposase or similar source. Thus, mutant phenotypes and enhancer traps identified by *P*-element based insertional mutagenesis (Cooley et al. 1988, Spradling et al. 1995) could have been maintained in next generations. Recently, the limitations of *P* elements to *Drosophila* species (Handler 1993) was overcome by the identification of broad-range TEs of Class II (reviewed by Handler and James 2000) and caused breakthrough in insect transgenesis via germ-line transformation. However, simultaneously, it brought higher risks that the broad-range TEs will be cross mobilized by the transposase source of the same family in host species or horizontally transmitted to another one. This must be considered especially when transgenic species are introduced into nature. Based on that, the non-autonomous character of TEs for stable germ-line transformation is, in regard to further applications, insufficient and its reliable stabilization is required. One of the proposed strategies to stabilize TEs are chromosomal rearrangements by using the site-specific recombination within a single or two TEs, resulting in deletion or inversion of terminal inverted repeats (TIRs) (Handler 2004) and prohibiting TE from remobilization.

In this thesis, the broad-range TEs, *piggyBac* and *Hermes*, were analysed. TEs contained promoter and fluorescent marker that were separated by the target site of FLP/*FRT* site-specific recombination system, so that inversion between *FRT*s of two TEs resulted in the exchange of their TIRs. Inversions that contained exchanged TIRs between two TEs: (1)

piggyBac/piggyBac on X chromosome and (2) between *piggyBac*/*Hermes* on the third chromosome of *D. melanogaster*, were analyzed and in both cases, efficient stabilization was observed. The first case is the additional verification that *piggyBac* TE needs both intact 5' and 3' TIRs (Elick et al.1997, Li et al. 2001, Handler et al. 2004). To this contributed also the second case of rearranged *piggyBac*/*Hermes*. However, this latter one brought a new proof that *Hermes* TE needs both 5'TIR and 3'TIR as well. This is especially important, because the cross mobilization of *Hermes* TE by the broad-range TE, *hobo*, was observed in *D. melanogaster* (McGinnis et al. 1983). Moreover, 5'TIR or 3'TIR of *Hermes* TE can not be replaced by the TIRs of *piggyBac* TE and vice versa, which fulfilled expectations as a reason that TIRs of *Hermes* and *piggyBac* differ on their sequence level, since these two TEs belong to different transposon families.

In sum, TIR rearrangements of TEs can provide the stabilization of integrated TEs in host species and protect against their remobilization, which would result in unexpected genomic modifications with unknown impact on host species nature.

4.3.3 Deletions and duplications

Duplication and deletion of ca. 56 kb region were successfully established in *trans* position on the left arm of the 3rd chromosome in *D. melanogaster*. *FRT* sites were placed in non-P-element vectors, one in *Hermes* and the other in *piggyBac*. This suggested that FLP is able to recognize *FRT* sites and mediate the site specific recombination without being influenced by *FRT* flanking sequences of: (i) fluorescent markers, (ii) 3xP3 and PUb promoters and (iii) *piggyBac* and *Hermes* terminal inverted repeats.

The creation of duplications and deletions was only in one out of two set ups successful. Reason for this might be plentiful.

Firstly, *FRT* sites could be susceptible to position effect of chromosomal conformation, surrounding insertion sites. In the successful case, *FRT* sites were inserted in genes while in the other case, in non-coding regions. This could reflect that euchromatic regions are likely better accessible for FLP recombinase than heterochromatic ones. Although negative result was obtained for the more distant combination, the ca. 160kb can not be the problem as much larger deletions could be created in *trans* position in *Drosophila* genome (Golic and Golic, 1996). This is also substantiated by the fact that this region is comprised in a confirmed deficiency, which was created by using the isogenic DrosDel Kit (http://www.drosdel.org.uk).

Secondly, a low frequency (around 1%) of ca. 56 kb deletion can point out that one may expect also lower frequency for the ca. 160 kb. Thus, if observed negative result was only due to the low frequency, the meiotic recombination of FRT sites on one chromosomal arm, i.e. *cis* position, could result in planned chromosomal rearrangement. Golic and Golic (1996) showed

that *FRT* sites in *trans* position are not as effectively recombined as those in *cis* position. Another possibility may be to carry out this experiment with doubled *hsp70*-FLP source to provide more FLP recombinase.

Finally, transmission of chromosomal rearrangement of longer deletion might not have been allowed through the germ-line due to a meiotic drive (e.g. Golic 1990, Spencer 2002). A possibility that the transmission of dyads was limited, might be supported by the fact that, interestingly, the duplication of ca. 56 kb was always accompanied by its deletion but not vice versa and considerably fewer males contained this deletion than the duplication. A disadvantage of deficiencies is likely related to missing genes that are normally present in the region between *FRT* sites and could be responsible for which cells produce viable gametes, giving rise to a fertile progeny. Therefore, an intense screen for somatic recombination, which was not evaluated here and is more demanding in regard to fluorescent markers, could demonstrate whether the deletion/duplication of ca. 160 kb region took place.

High sterility of single male crosses suggested potential severe effects of *hsp70* driven FLP in the case of ca. 56 kb region. Golic et al. (1997) showed that *hsp70*-FLP is less effective than β*2-tub*-FLP, thus, to get higher duplication/deletion frequency for this region and to increase a chance of rearrangements even for ca. 160 kb region, it would be worth using β*2-tub*-FLP source. Though, this promoter is not effectively active before homologous chromosome segregation, i.e. before onset of anaphase of meiosis I during spermatogenesis (Golic et al. 1997). Thus, only insufficient amounts of FLP are mediated pre-meiotically and majority of mRNA is translated after meiosis I as suggested by Golic et al. (1997). Although both *FRT* sites could be recombined onto one chromosomal arm, the simple and elegant point of creating defined deletions/duplications without the necessity of this additional recombination to get *cis* position of *FRT*s vanishes. β*2-tubulin* promoter may be considered to be alternatively applied for a FLP-mediated recombination between e.g. heterologous chromosomes. Beumer et al. (1998) referred that such site-specific recombination by using *hsp70*-FLP results in translocation events in *D. melanogaster*.

In sum, the successful establishment of defined deletion/duplication by using the broad-range TEs, *piggyBac* and *Hermes*, as well as the yeast FLP/*FRT* recombination system in *D. melanogaster* suggests to further test this system for its functionality in the red flour beetle, *T. castaneum*. The easy identification of designed chromosomal rearrangements will be certainly of advantage, especially due to the fact that only few chromosomal markers and no evidence of endoreduplicated tissues in *T. castaneum* are available (Brown et al. 2003). Additional advantage of such chromosomal rearrangements is that the limitations, caused by haplo-insufficient genes (Ashburner 1989), can be obviate when a defined suitable duplication to a deletion in the chromosomal region is provided.

5 SUMMARY

The discovery of non-species-specific, broad-range transposable elements and the establishment of a universal 3xP3 promoter revolutionized insect transgenesis. It overcame the limitations of the germ-line transformation to be restricted to the model organism *Drosophila melanogaster*. In combination with discernable fluorescent markers, multi-component systems, such as transposon-based insertional mutagenesis, can now be introduced to various insect species.

To drive the *transposase* gene for transposable element remobilisation, suitable promoters are needed. The broadly conserved thermotolerance factor, Hsp70, is well-characterised in *D*. *melanogaster* and its promoter, which is inducible by high temperatures, provides a genetic tool for transient gene activation. In this thesis, I could prove that the *D*. *melanogaster hsp70* promoter is functional in *Tribolium castaneum* as well. Its observed basal level activity, however, must be considered and limits its use for experiments, where no strict transient gene expression is required. Nevertheless, the *D*. *melanogaster hsp70* promoter will suffice to provide an efficient transposase source in transposon-based mutagenesis screens in *T*. *castaneum*.

The remobilization of non-autonomous transposable elements in such screens results in novel mutations and tagging of potentially interesting *cis*-regulatory elements. To further investigate gene functions, misexpression studies are necessary. In *D*. *melanogaster*, this can be done by directed binary expression systems. Here I could show, that the combination of Gal4Δ/*UAST* works best in *D*. *melanogaster* somatic tissue, whereas the LexA/$(LL)_4$ and the tetracycline-controlled systems seem to function only poorly. All constructs are based on broad range transposons as well as universal markers and promoters, so that they can be used in other insect species to determine the best system. Preliminary tests in *T*. *castaneum*, however, showed that there are a number of additional problems that need to be addressed, before a suitable binary expression system can be established for this species.

The full genome sequence of *T*. *castaneum* is now available. Therefore, interesting mutations, *cis*-regulatory elements and their biological functions can be directly linked to the sequence level. When target sites of site-specific recombination systems are included in insertional mutagenesis screens, their insertion sites can be precisely identified and designed chromosomal rearrangements (inversions, duplications and deletions) created. Here I could present a universal system, which can be introduced into non-drosophilid species and enables such chromosomal rearrangements, which I could successfully demonstrate in *D. melanogaster*. Defined inversions suppressed meiotic recombination between inverted and non-inverted regions on homologous chromosomes and can thus serve as defined balancer chromosomes. Also defined deletions/duplications were generated in *D*. *melanogaster*. Such

aberrations will be crucial in other insect species, like *T. castaneum*, to safely keep mutation stocks and identify gene functions. Moreover, the separation of terminal inverted repeats by inverting the chromosomal region between two transposable elements resulted in immobilization. This is of a particular interest for applied transgenesis approaches in insect pest management, when transgenic insects will be released into the nature and transposable elements must be efficiently protected from potential cross mobilization in host species.

6 ZUSAMMENFASSUNG

Die Entdeckung nicht-speziesspezifischer Transposons mit breitem Wirtsspektrum und die Etablierung des artifiziellen Promotors 3xP3 revolutionierte die Möglichkeiten zur Transgenese von Insekten. Damit konnte die Begrenzung der Keimbahntransformation auf die Taufliege *Drosophila melanogaster* überwunden werden. In Kombination mit unterscheidbaren, fluoreszierenden Markern können nun Multikomponentensysteme, wie die Transposon basierte Insertionsmutagenese, in verschiedene Insektenarten eingeführt werden.

Um das Tranposasegen für die Remobilisierung von nicht-autonomen Transposons zu exprimieren, werden geeignete Promotoren benötigt. Der phylogenetisch konservierte Thermotoleranzfaktor HSP70 ist in *D. melanogaster* gut untersucht, und der zugehörige Promotor, der bei hohen Temperaturen aktiviert wird, bietet ein genetisches Werkzeug für die konditionelle Genexpression. In dieser Arbeit konnte ich nachweisen, dass der *D. melanogaster hsp70* Promotor auch im Mehlkäfer *Tribolium castaneum* funktioniert. Die beobachtete basale Aktivität des Promotors muss jedoch in Betracht gezogen werden und beschränkt dessen Verwendung für Experimente, bei denen die basale Aktivität nicht stört. Dennoch eignet sich der *D. melanogaster hsp70* Promotor für die Expression von Transposasegenen bei der Transposon basierten Mutagenese von *T. castaneum*.

Die Remobilisierung von nicht-autonomen transponierbaren Elementen in solchen Durchmusterungsexperimenten führt zu neuen Mutationen und dem Aufspüren von interessanten *cis*-regulatorischen Elementen. Um Genfunktionen weiter zu untersuchen, benötigt man die Möglichkeit von Missexpressionsstudien. In *D. melanogaster* können dafür gesteuerte binäre Expressionssysteme herangezogen werden. Hier konnte ich zeigen, dass in solchen Systemen die Kombination von Gal4Δ/*UAST* im somatischen Gewebe am besten funktioniert, während LexA/$(LL)_4$ und die Tetracyclin kontrollierten Systeme nur schwach zu funktionieren scheinen. Alle verwendeten Konstrukte basieren auf Transposons mit weitem Wirtsspektrum, sowie universellen Markern und Promotoren, so dass sie leicht auf andere Insektenarten übertragen werden können, um auch dort die besten Systeme bestimmen zu können. Erste Untersuchungen in *T. castaneum* haben jedoch gezeigt, dass es eine Anzahl weiterer Probleme gibt, die angegangen werden müssen, bevor geeignete binäre Expressionssysteme in dieser Spezies zum Einsatz kommen können.

Seit Anfang dieses Jahres steht die vollständige Genomsequenz von *T. castaneum* zur Verfügung. Daher können interessante Mutationen oder *cis*-regulatorische Elementen und ihre biologischen Funktionen direkt mit der Gensequenz in Verbindung gebracht werden. Wenn mittels Insertionsmutagenese ortspezifische Rekombinaseschnittstellen im Genom verteilt werden, können die Insertionsstellen präzise identifiziert und designierte Rekombinationen (Inversionen, Duplikationen, Deletionen) hergestellt werden. In dieser Arbeit konnte ich ein

universelles System beschreiben, dass auch in andere nicht-drosophilide Arten eingeführt werden kann, um entsprechende Umstrukturierungen von Chromosomen vornehmen zu können. Die Vorgehensweise konnte ich in *D. melanogaster* erfolgreich demonstrieren. Definierte Inversionen unterdrücken meiotische Rekombination zwischen der invertierten und nicht-invertierten Region homologer Chromosomen und können daher als definierte Balanzierchromosomen dienen. Auch definierte Deletionen/Duplikationen wurden in *D. melanogaster* generiert. Solche Abberationen werden entscheidend dazu beitragen können, damit in anderen Insektenarten, wie *T. castaneum*, Mutantenstämme sicher gehalten bzw. Genfunktionen identifiziert werden können. Zudem ermöglicht die Trennung der terminalen, invertierten Sequenzwiederholungen von transponierbaren Elementen durch die Invertierung der chromosomalen Region zwischen zwei Transposons deren Immobilisierung. Dies ist von entscheidendem Interesse für die Anwendung von transgenetischen Ansätzen in der Insektenschädlingsbekämpfung, wenn transgene Insekten freigesetzt werden sollen und daher die transponierbaren Elemente effizient vor einer potentiellen Kreuzmobilisierung in der Wirtsspezies geschützt werden müssen.

7 LITERATURE

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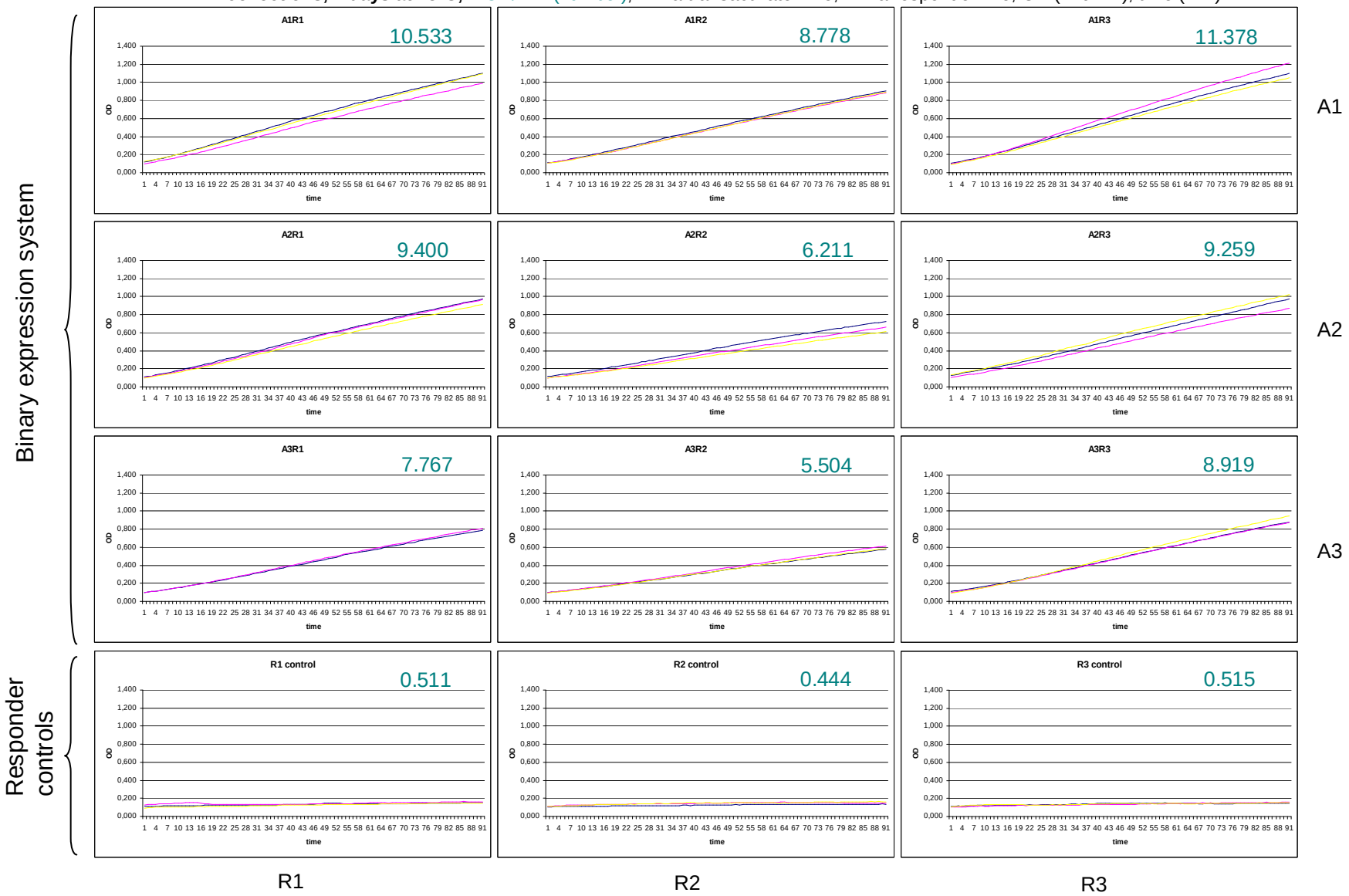
8 APPENDIX

APPENDIX A

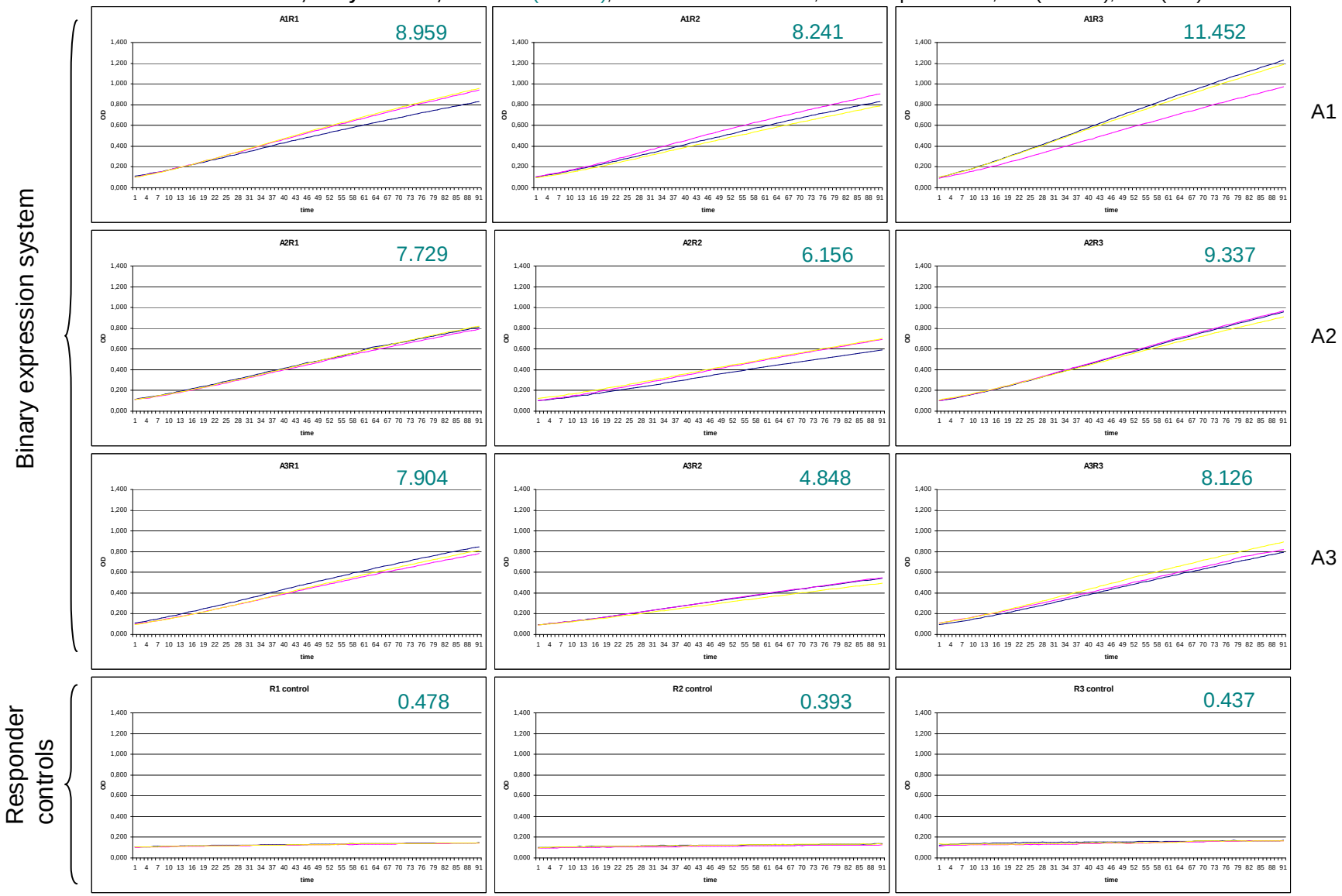
Drosophila melanogaster **Gal4*/UAST*** *first measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



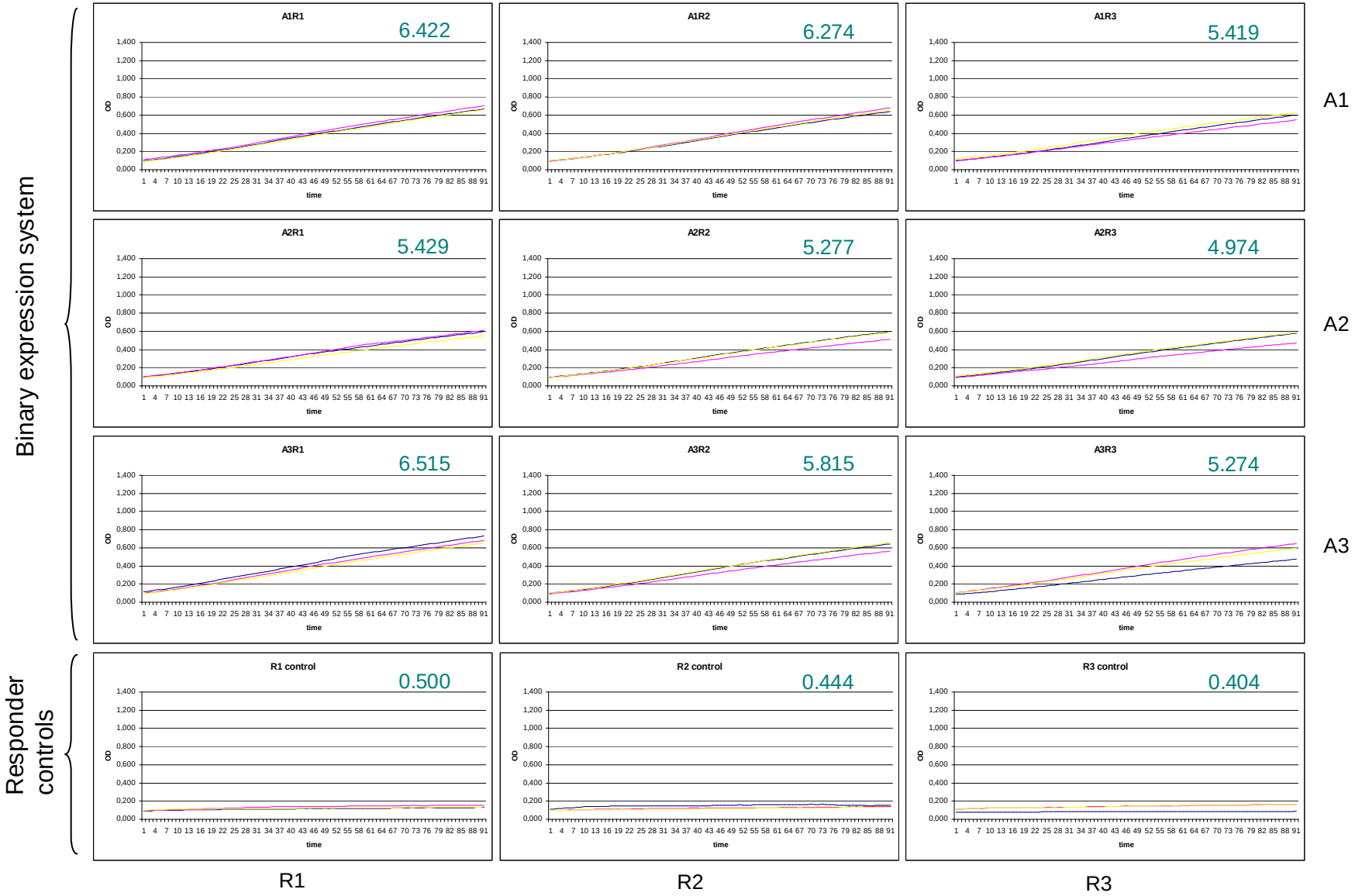
Drosophila melanogaster **Gal4/*UAST*** *second measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



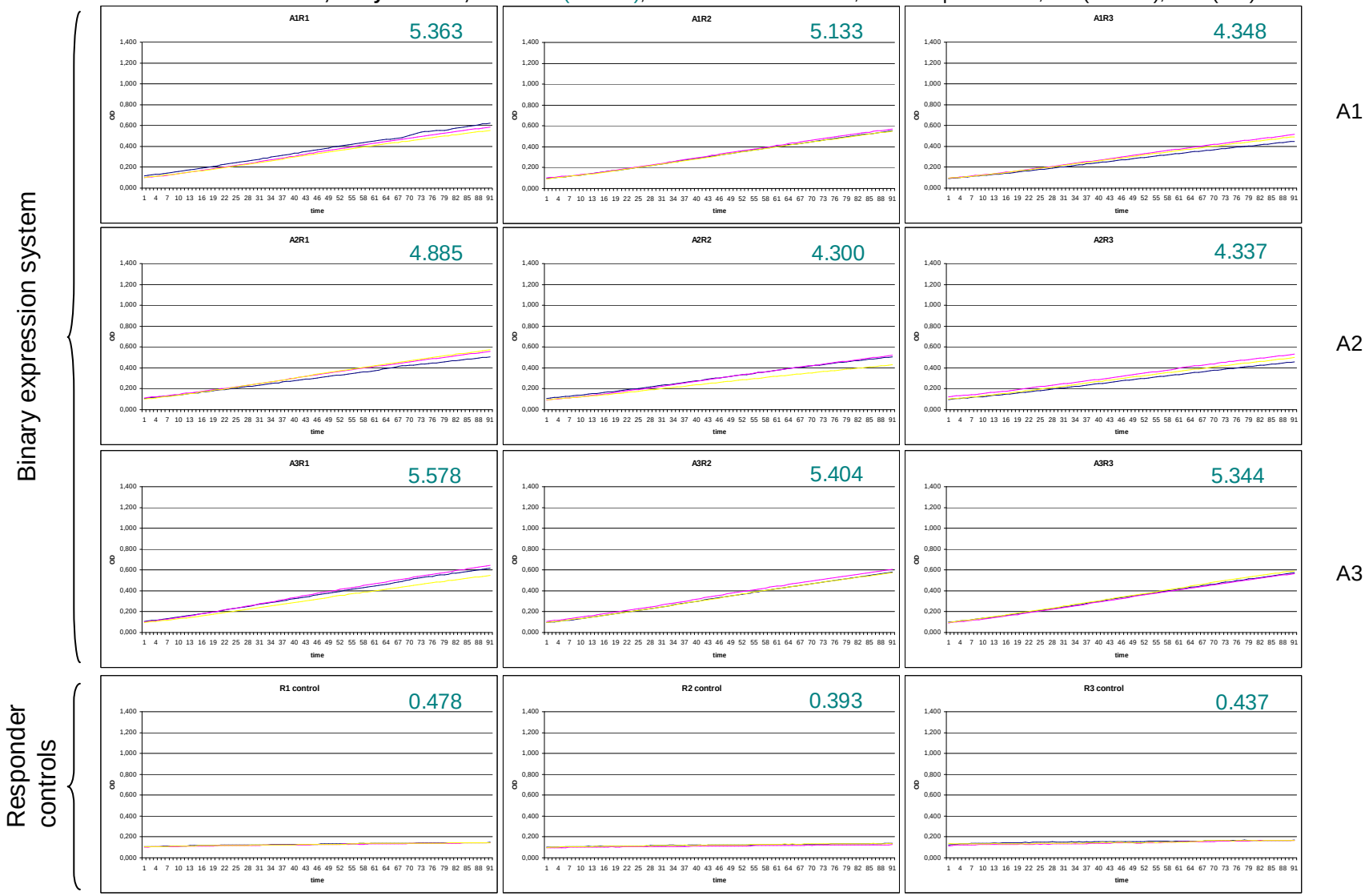
Drosophila melanogaster **Gal4/*UASp*** *first measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



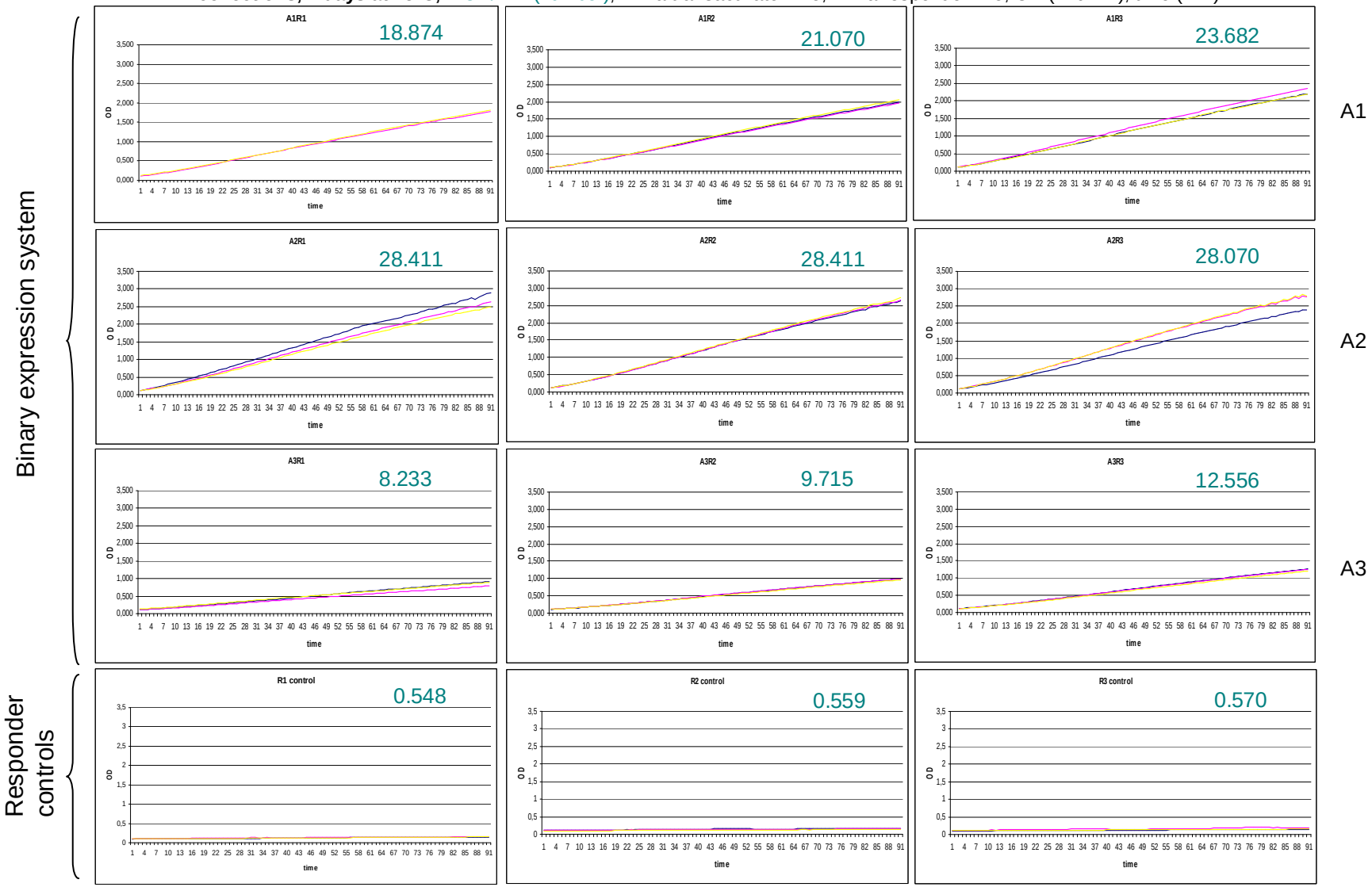
Drosophila melanogaster **Gal4/*UASp*** *second measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



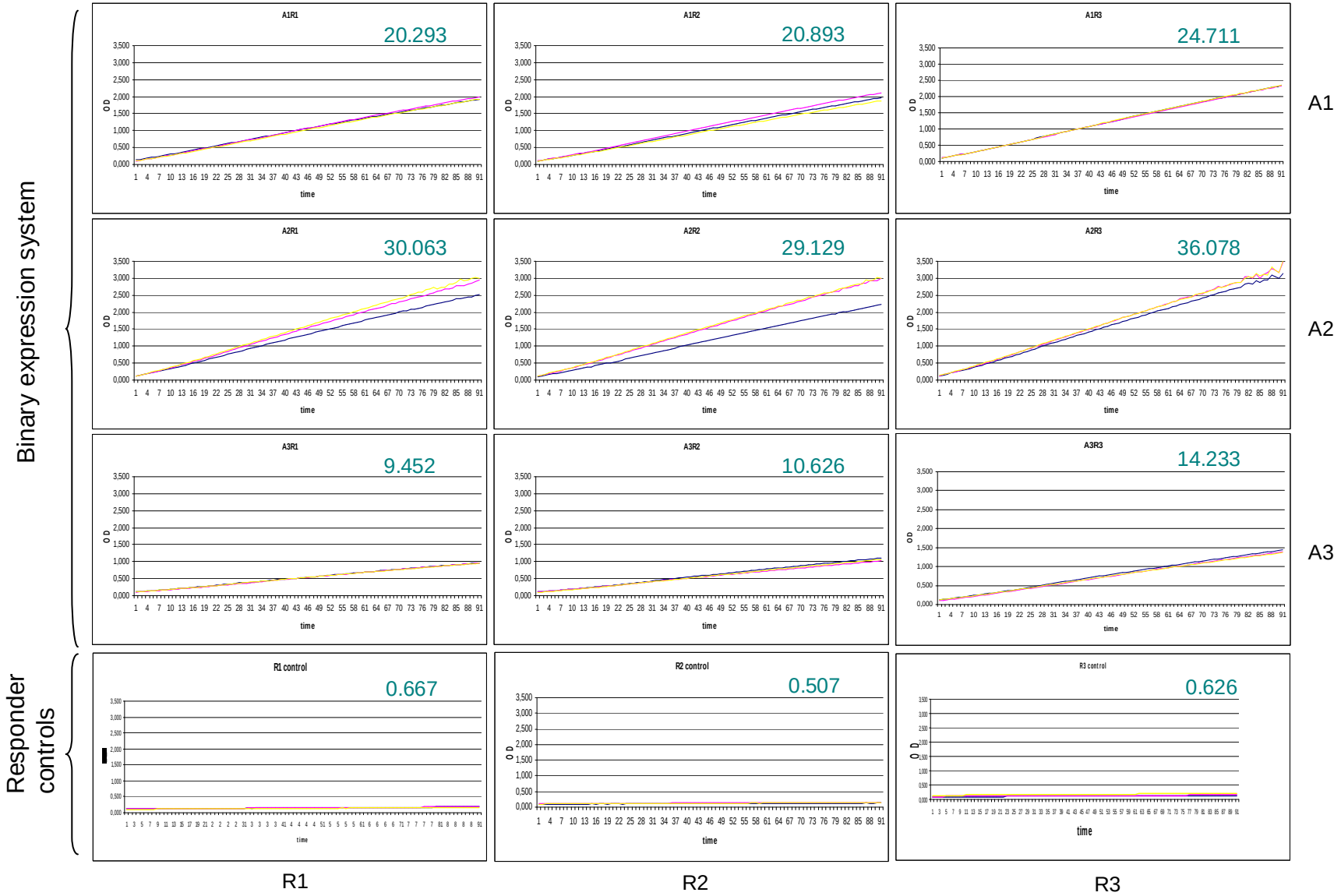
Drosophila melanogaster **Gal4Δ*IUAST*** *first measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



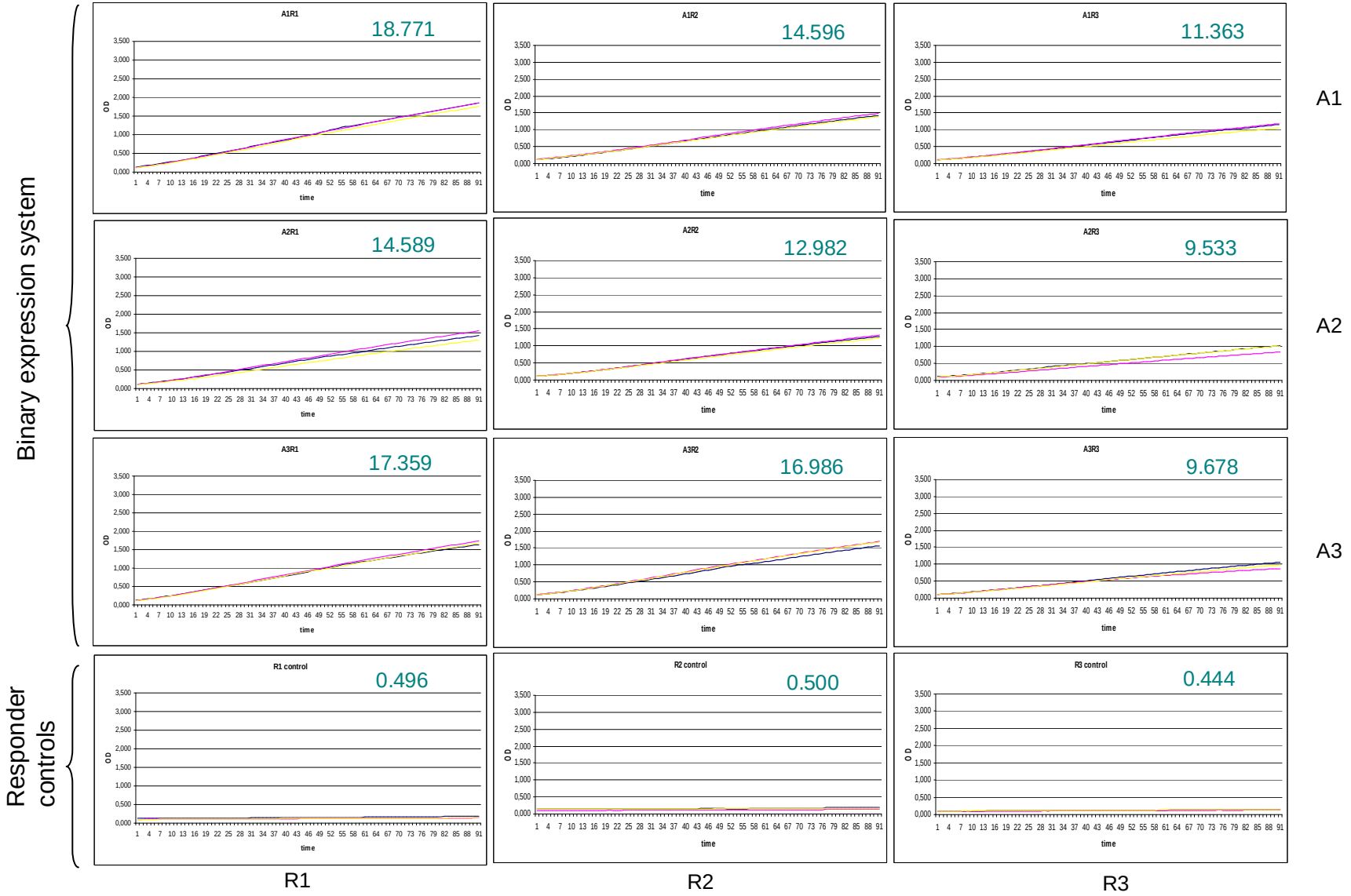
Drosophila melanogaster **Gal4Δ*IUAST*** *second measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



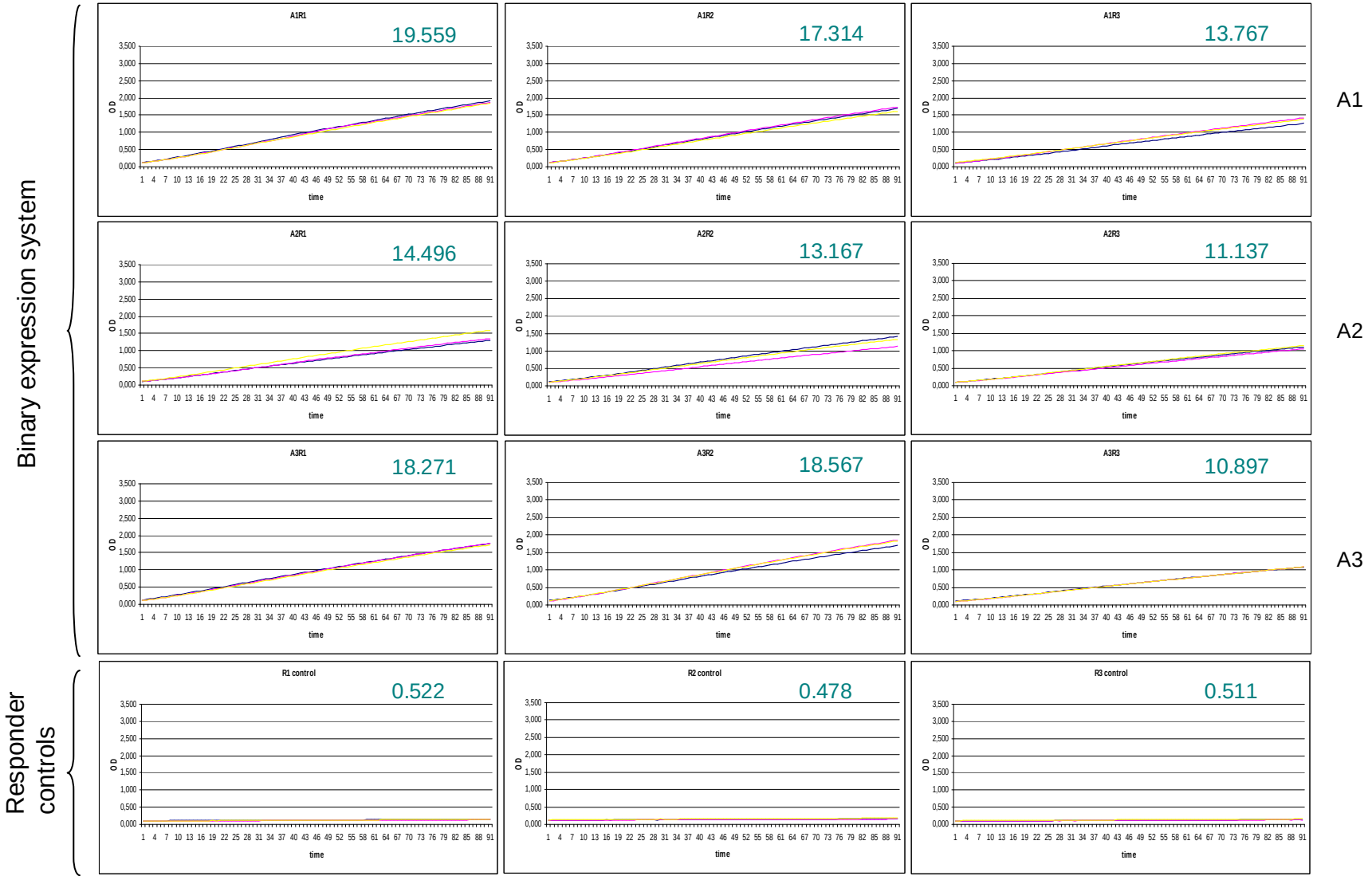
Drosophila melanogaster **Gal4Δ*lUASp*** *first measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



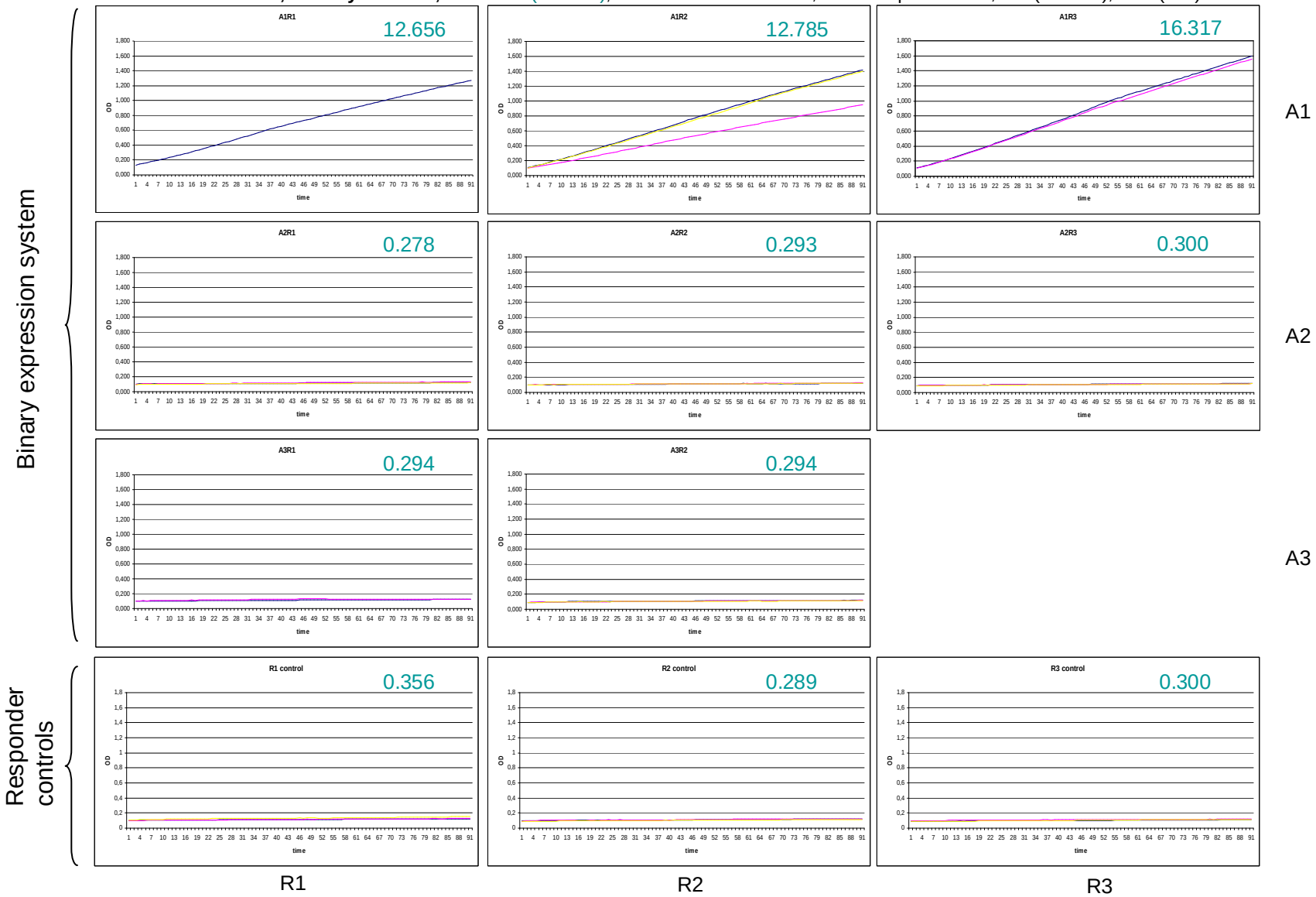
Drosophila melanogaster **Gal4Δ*UASp*** *second measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



Drosophila melanogaster **Gal4VP16*/UAST*** *first measurement*

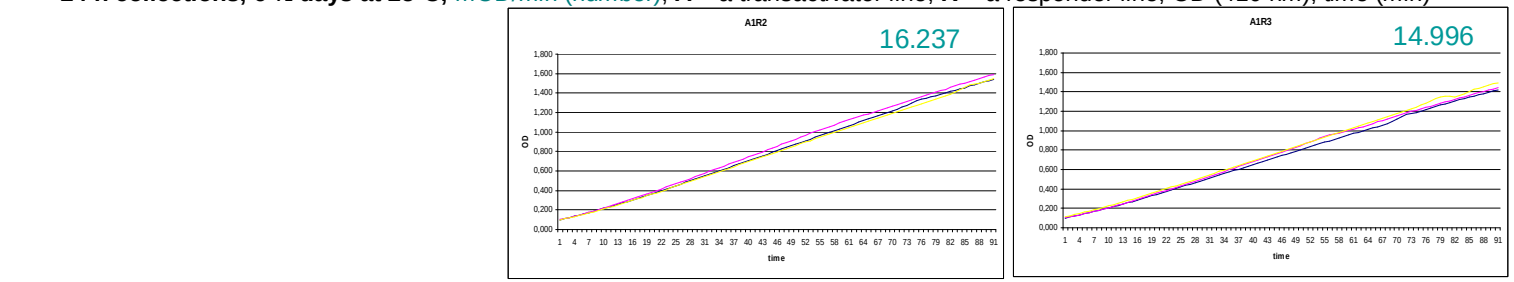
24 h collections, 6 ½ days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



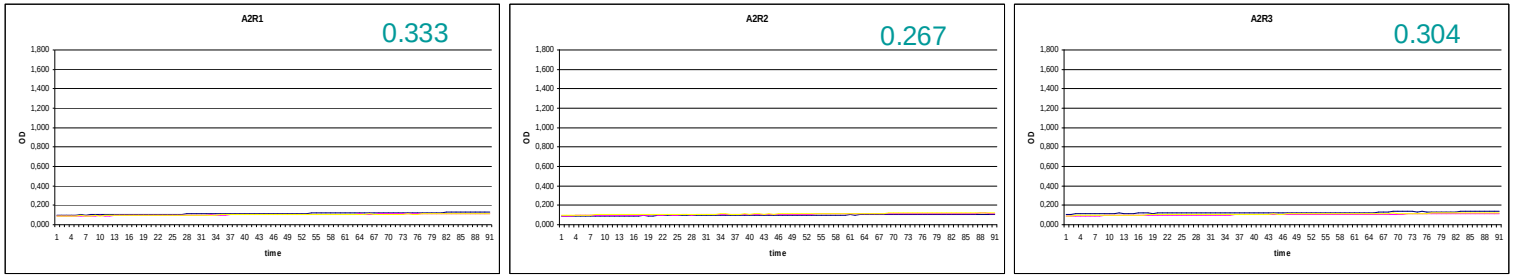
Drosophila melanogaster **Gal4VP16*/UAST*** *second measurement*

24 h collections, 6 ½ days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)

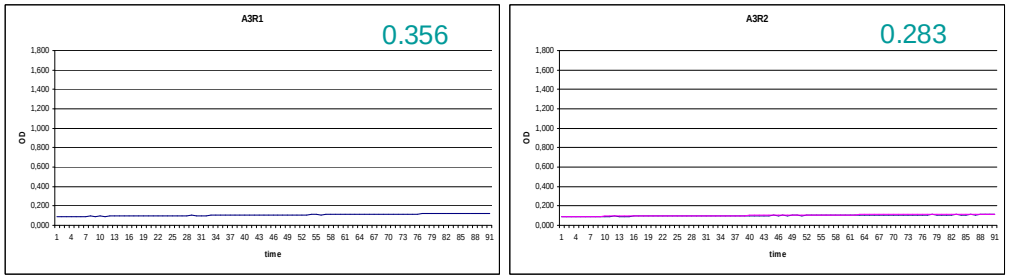
Binary expression system



A1

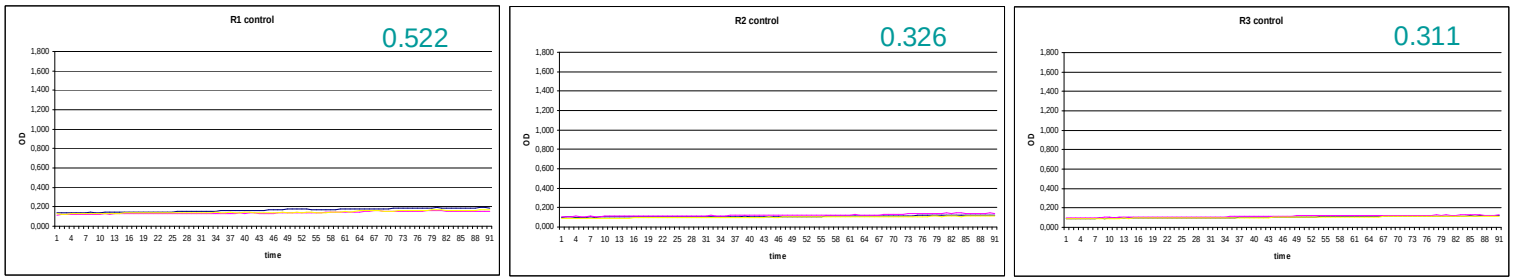


A2



A3

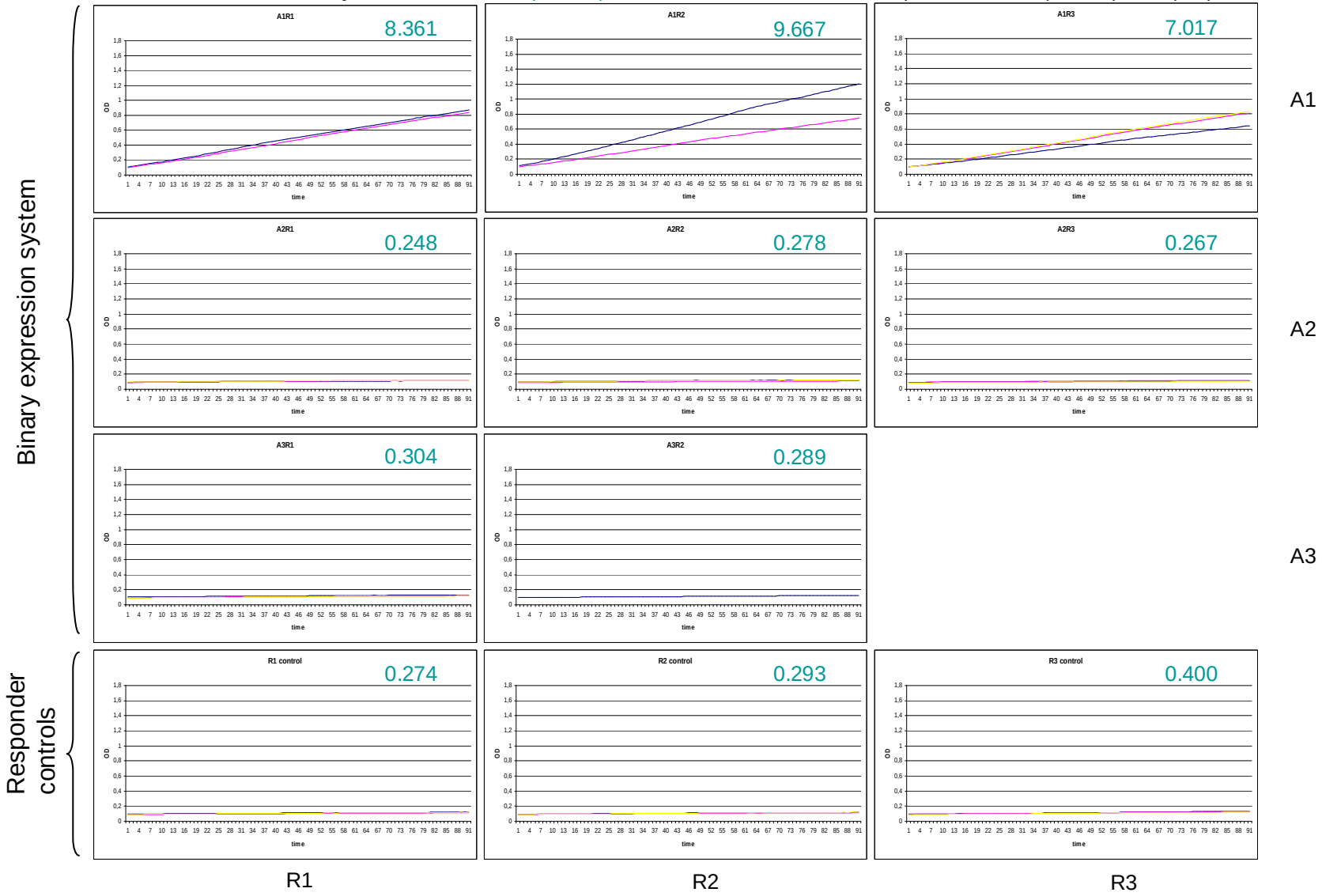
Responder controls



R1 R2 R3

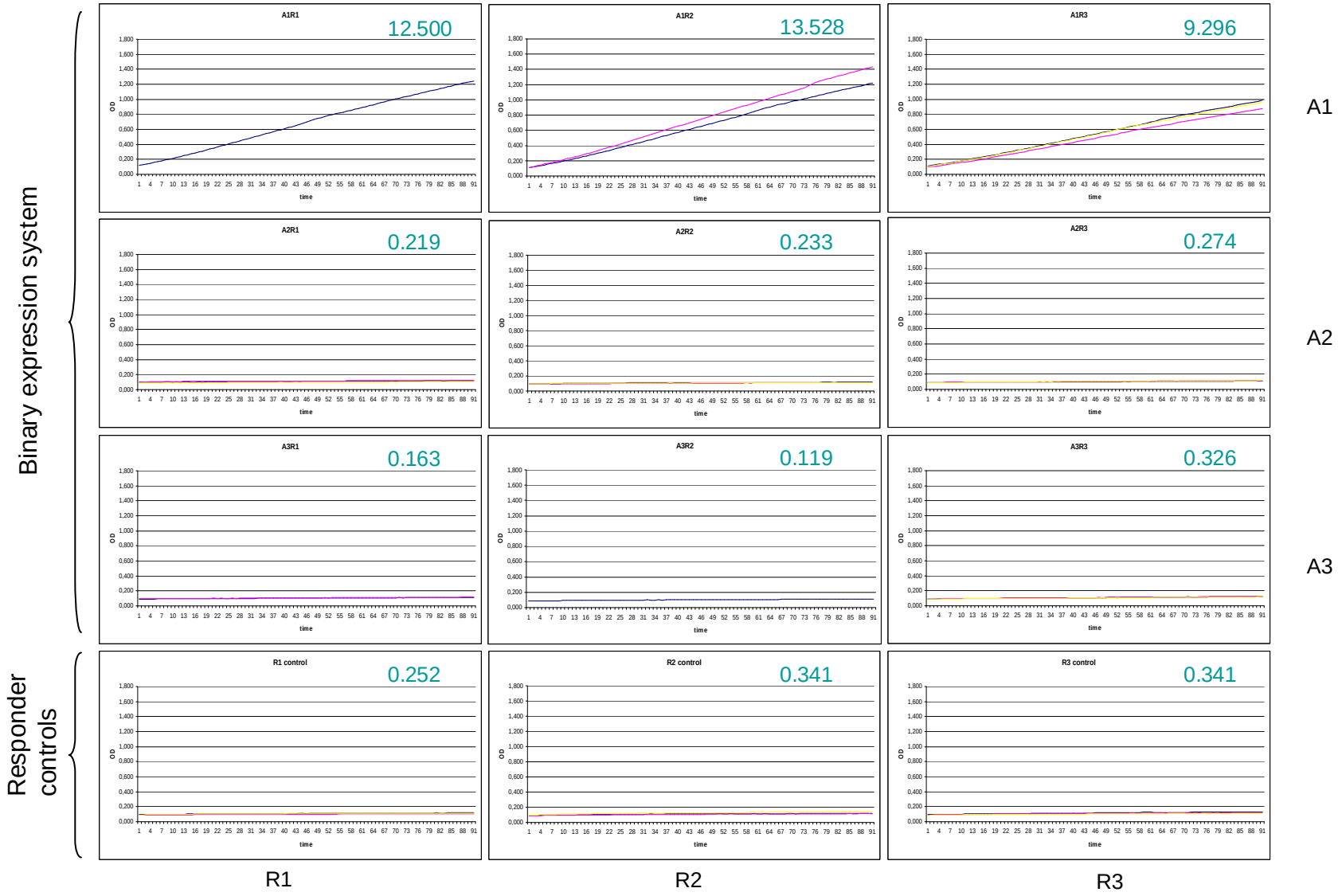
Drosophila melanogaster **Gal4VP16/*UASp*** *first measurement*

24 h collections, 6 ½ days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



Drosophila melanogaster **Gal4VP16/*UASp*** *second measurement*

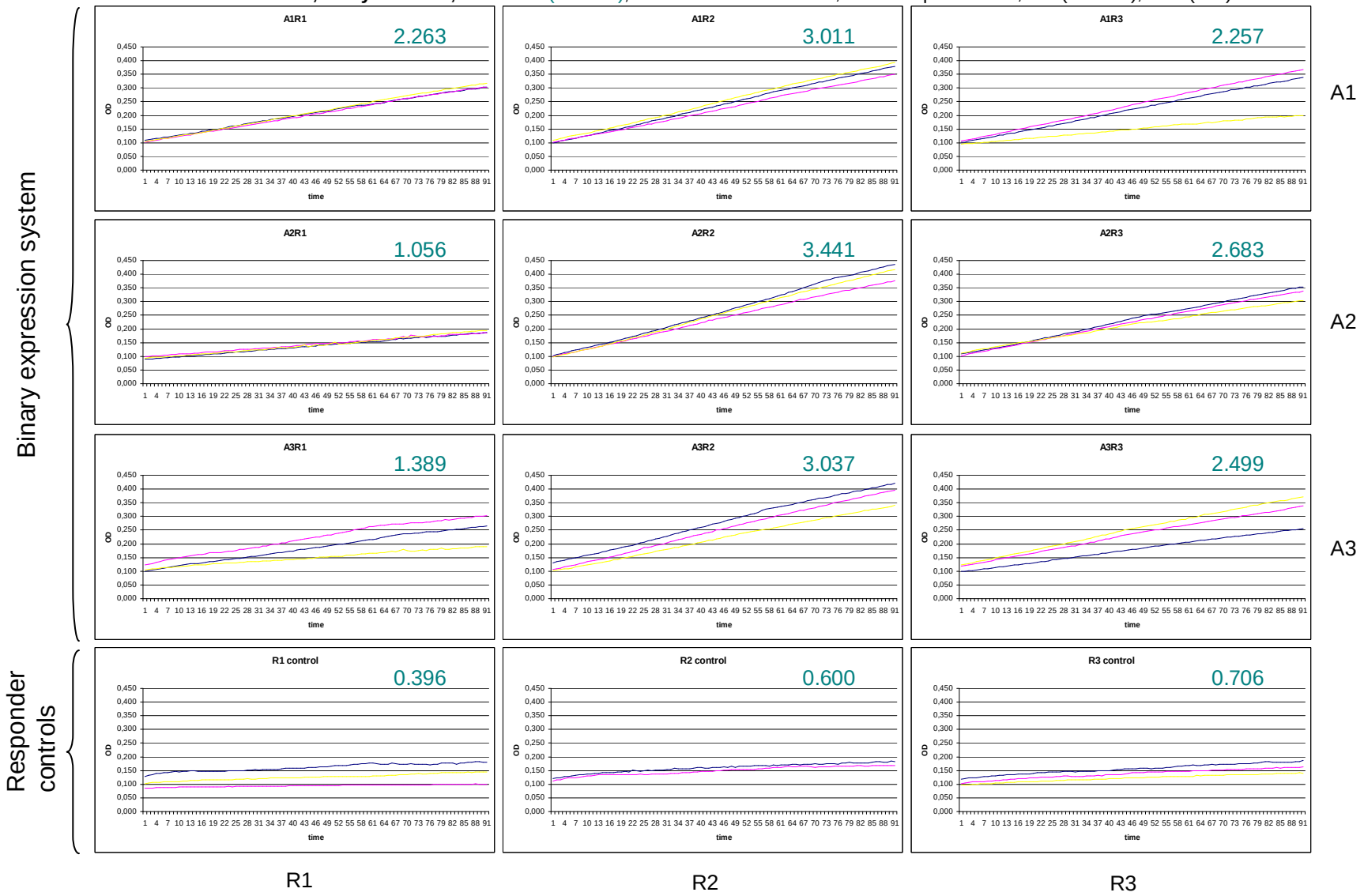
24 h collections, 6 ½ days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



APPENDIX B

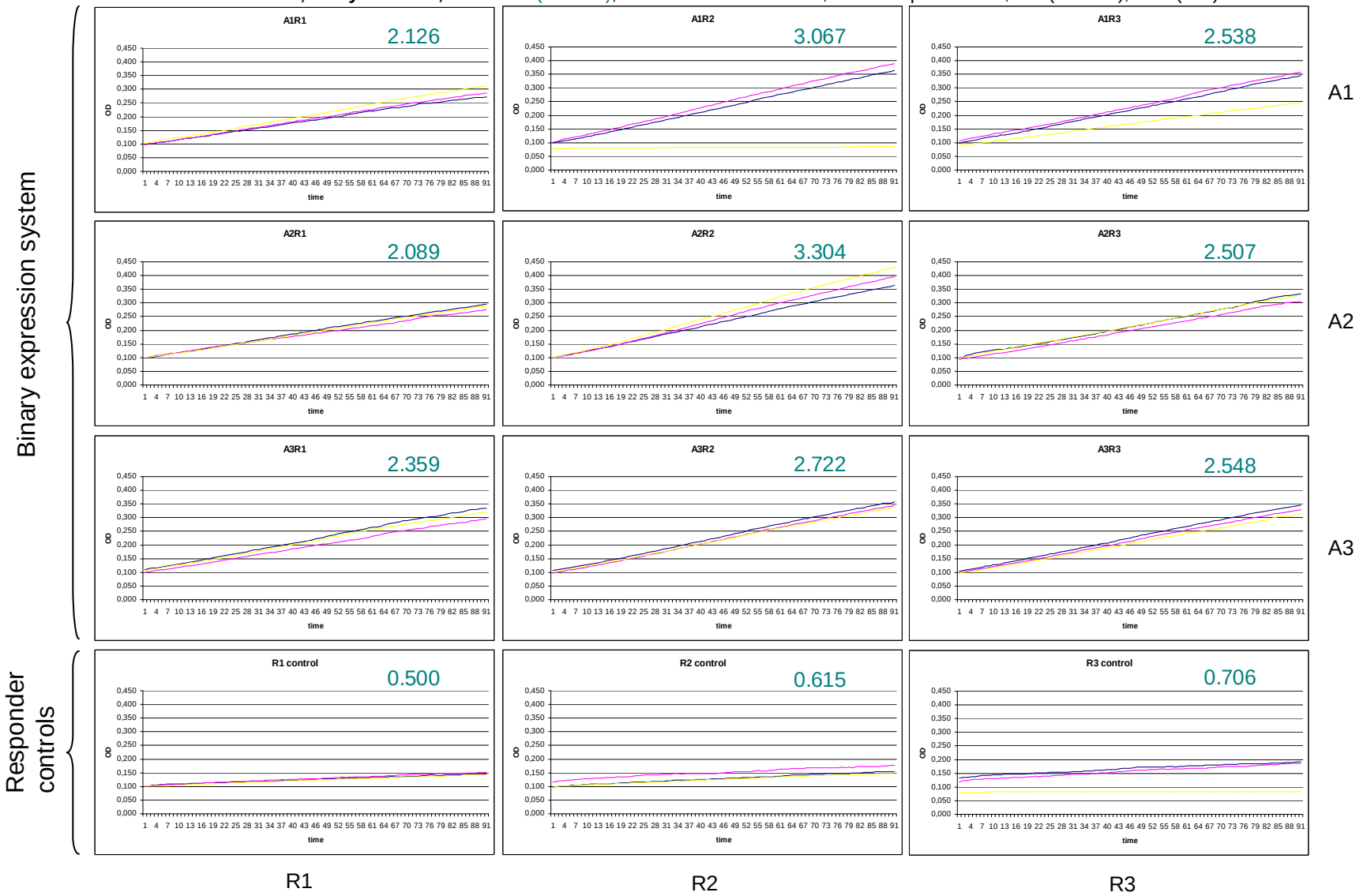
Drosophila melanogaster **LexA*I(LL)*$_4$** *first measurement*

10 h collections, 6 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



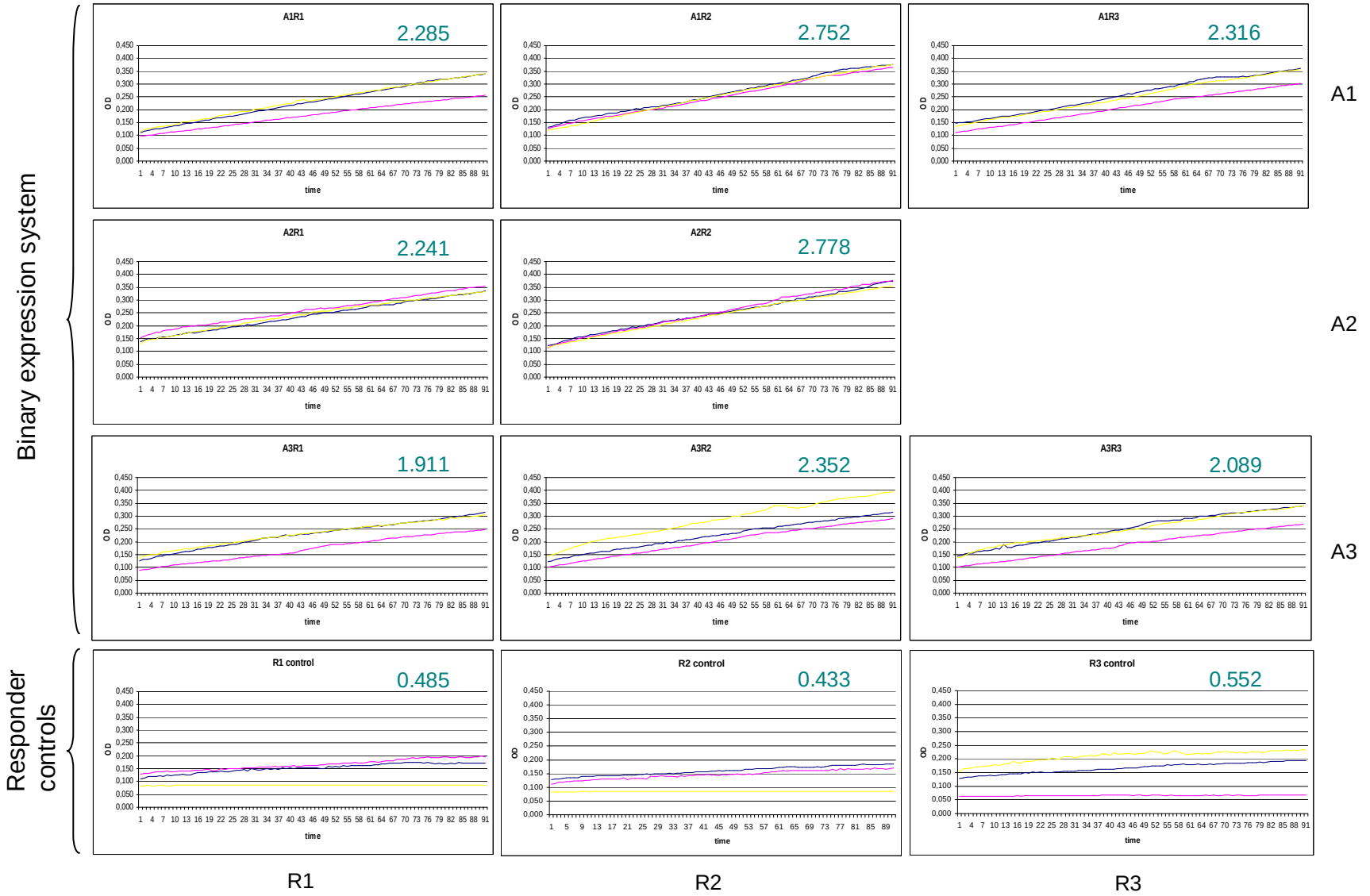
Drosophila melanogaster **LexA*I(LL)*$_4$** *second measurement*

24 h collections, 8 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



Drosophila melanogaster **LexA/(*LL*)$_4$** *third measurement*

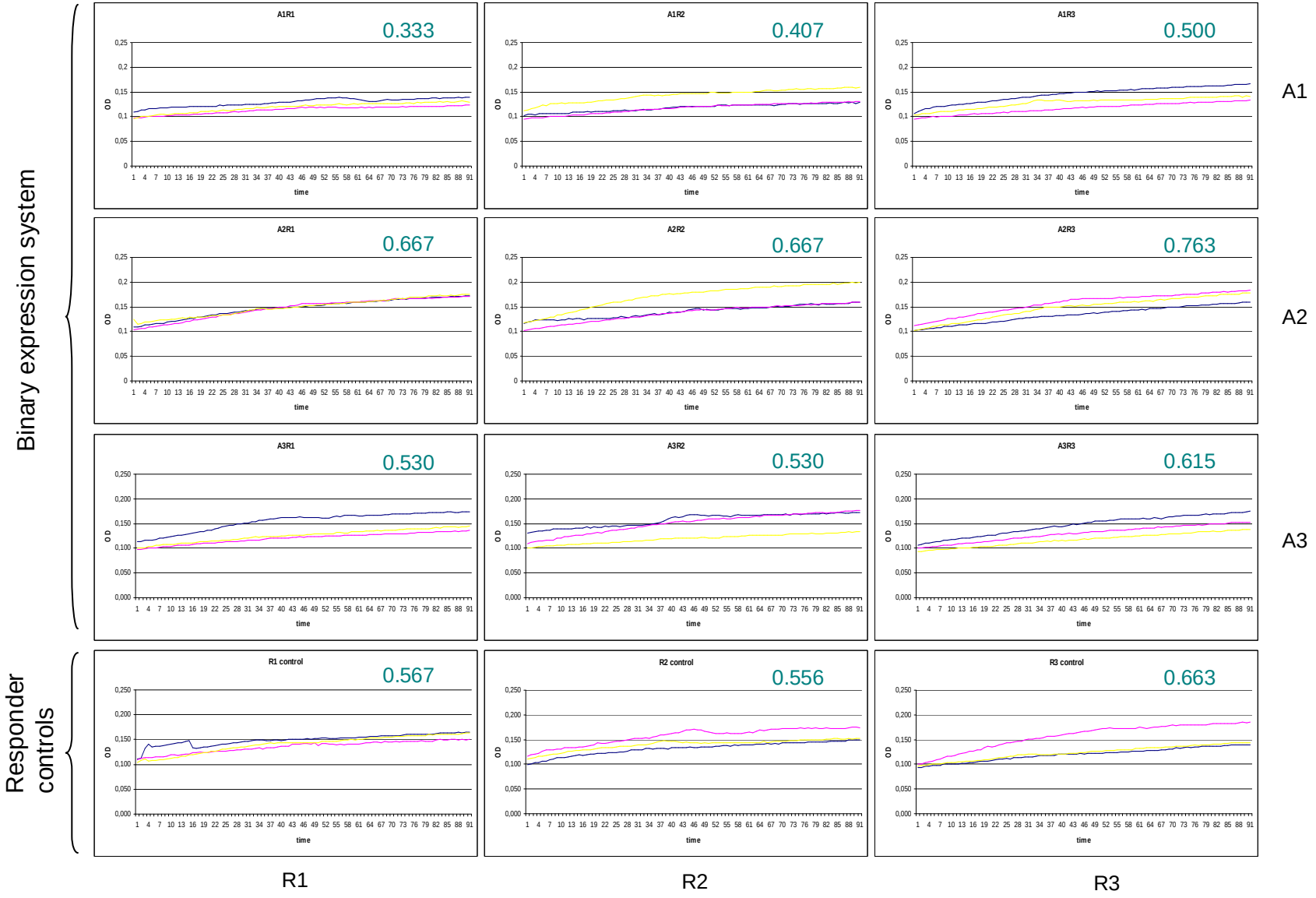
21 h collections, 27 days at 18ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



APPENDIX C

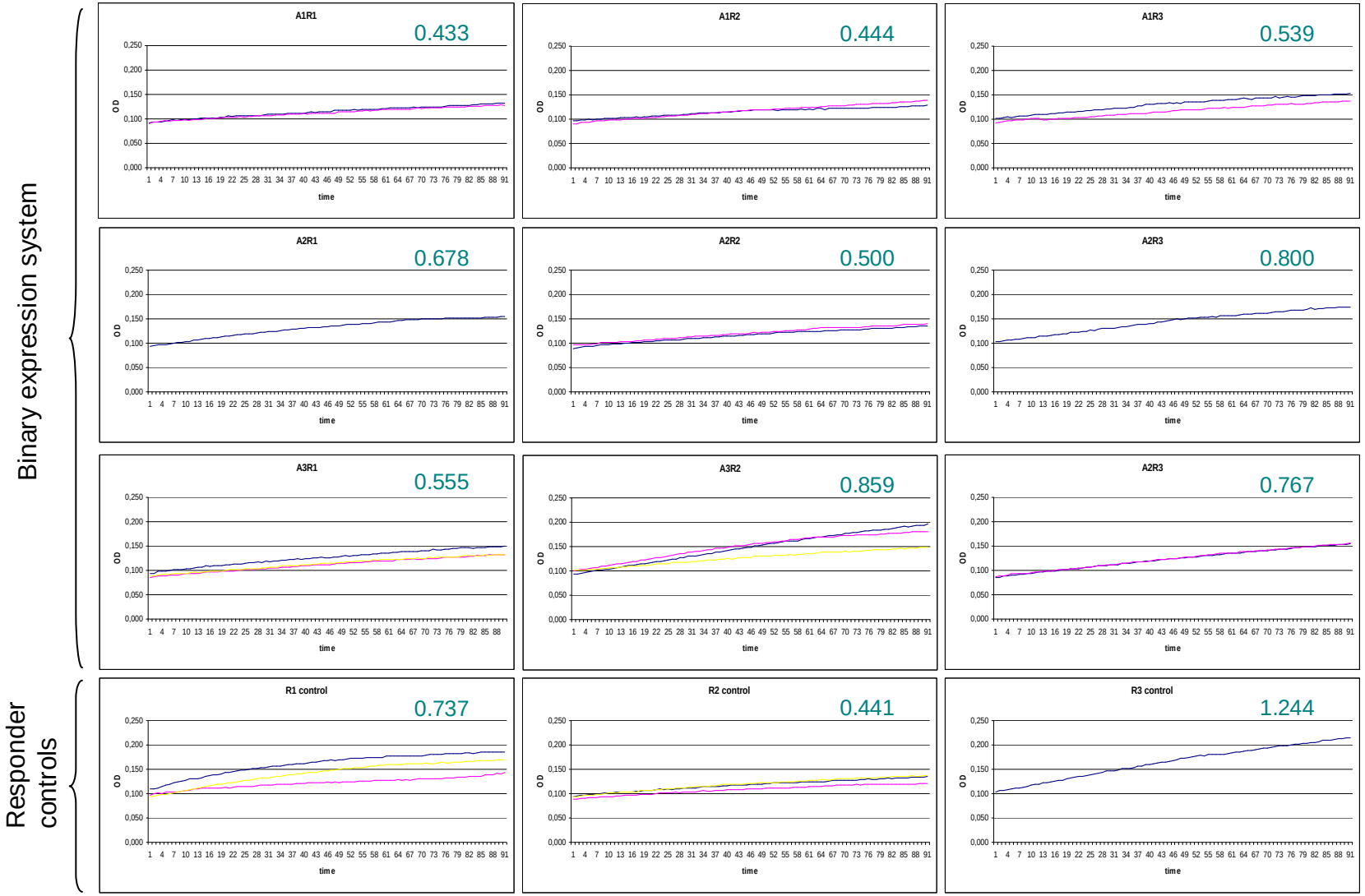
Drosophila melanogaster **tTA*ITRE*** *first measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



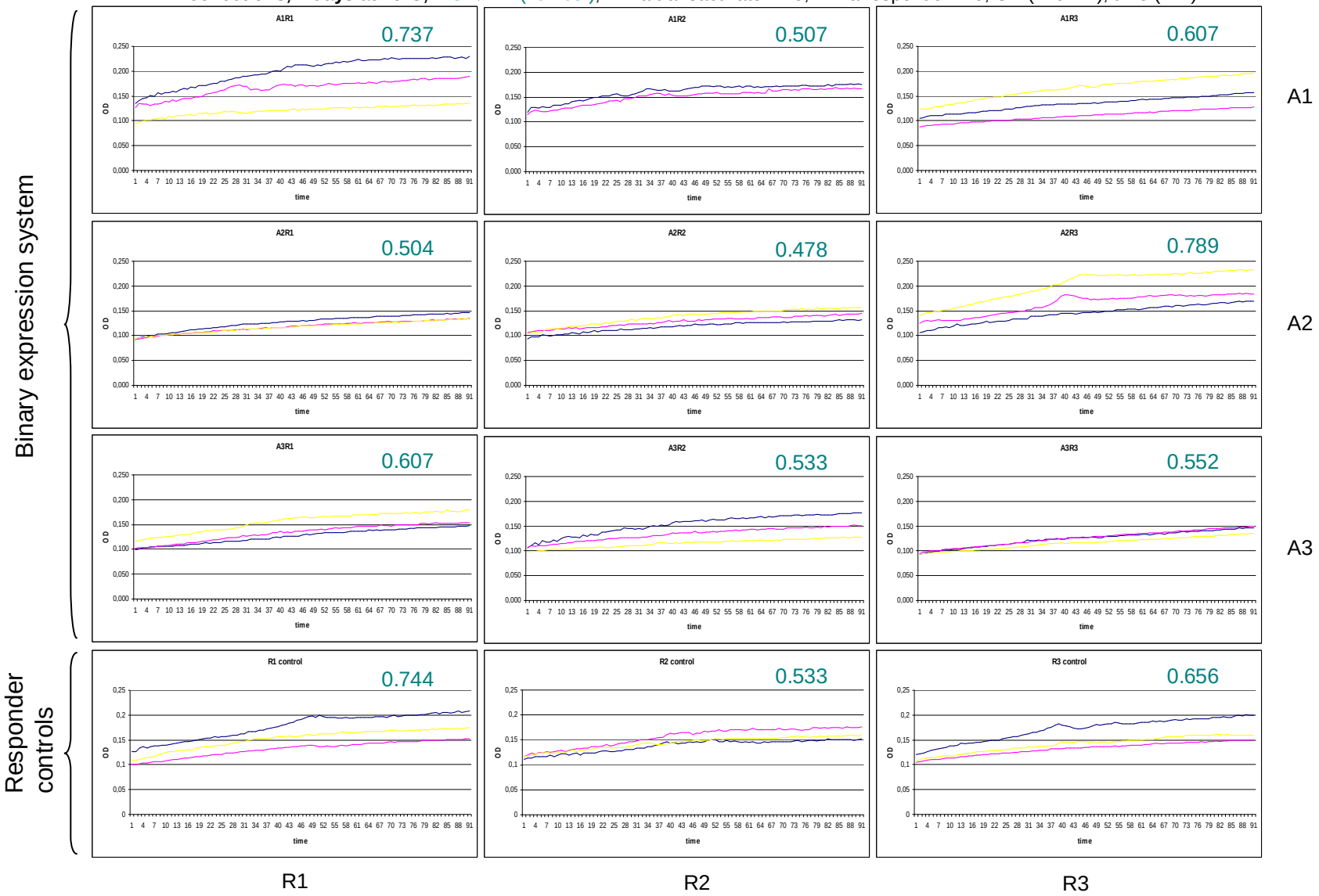
Drosophila melanogaster **tTA*ITRE*** *second measurement*

24 h collections, 7 days at 25ºC + 20 days at 18ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



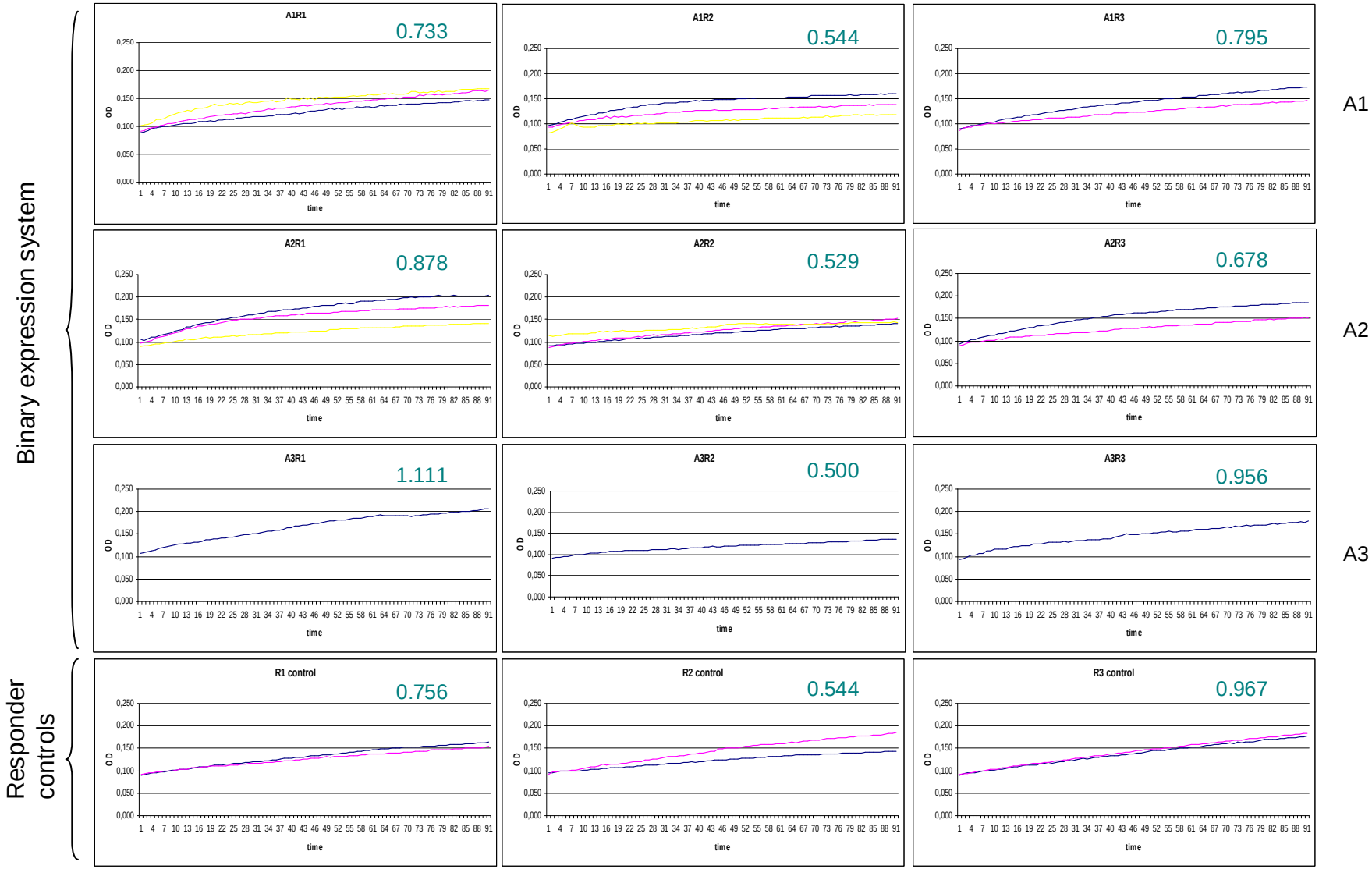
Drosophila melanogaster **sctTA*ITRE*** *first measurement*

24 h collections, 7 days at 25ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



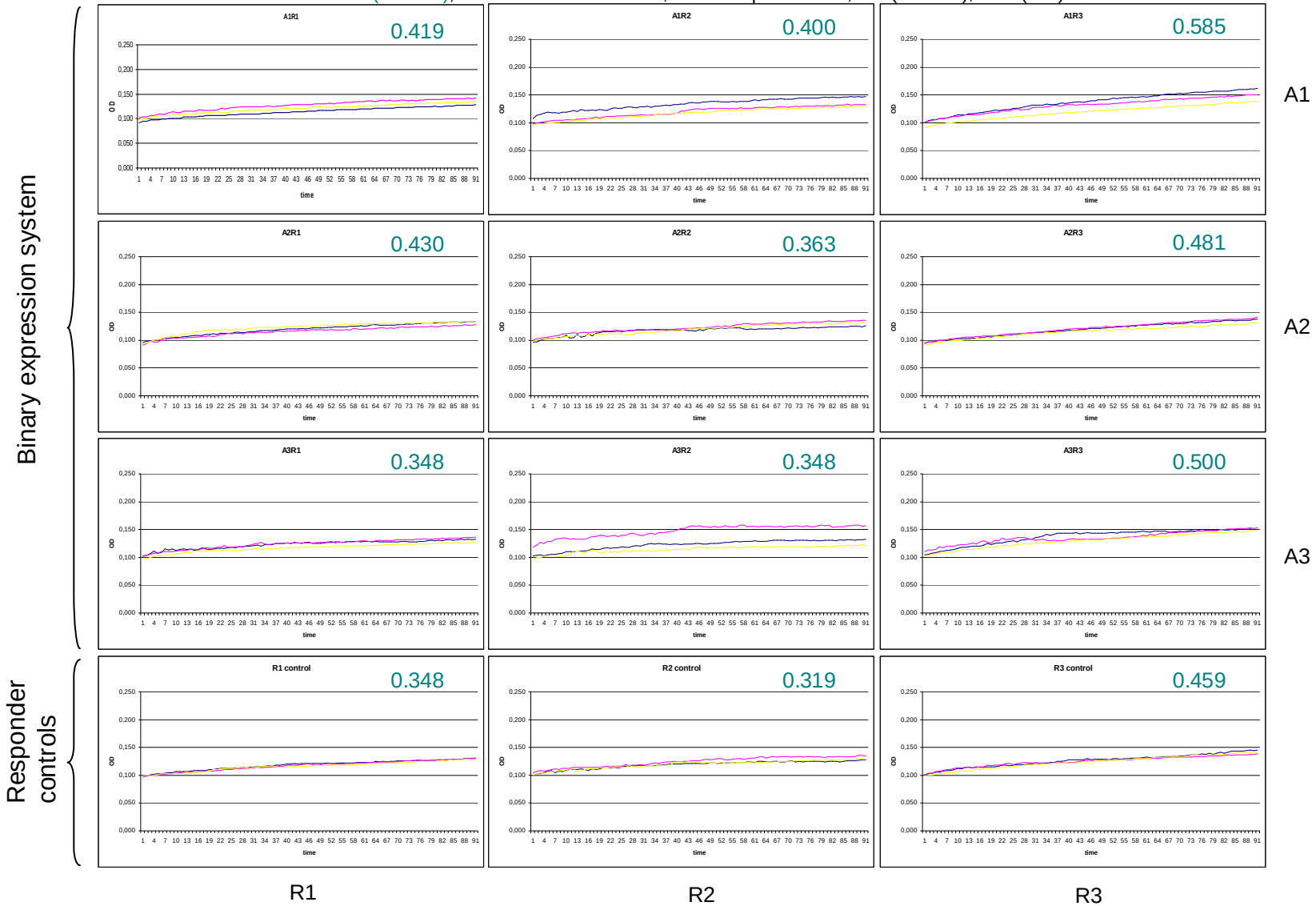
Drosophila melanogaster **sctTA*I TRE*** *second measurement*

24 h collections, 7 days at 25ºC + 20 days at 18ºC, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



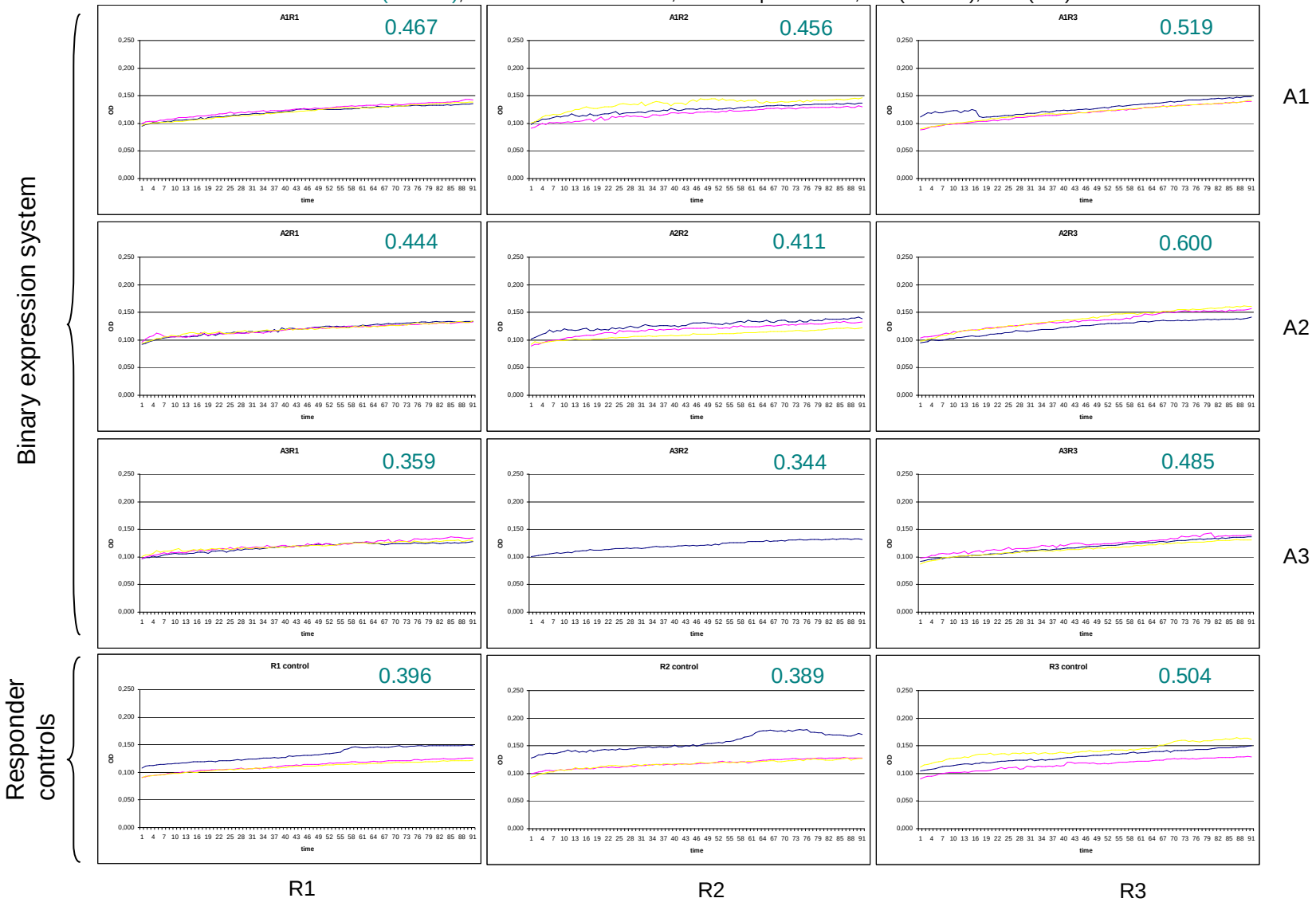
Drosophila melanogaster **scrtTA/*TRE*** *first measurement*

24 h collections, 7 days at 25ºC, food with tetracycline (100 µg/ml) in the dark,
mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



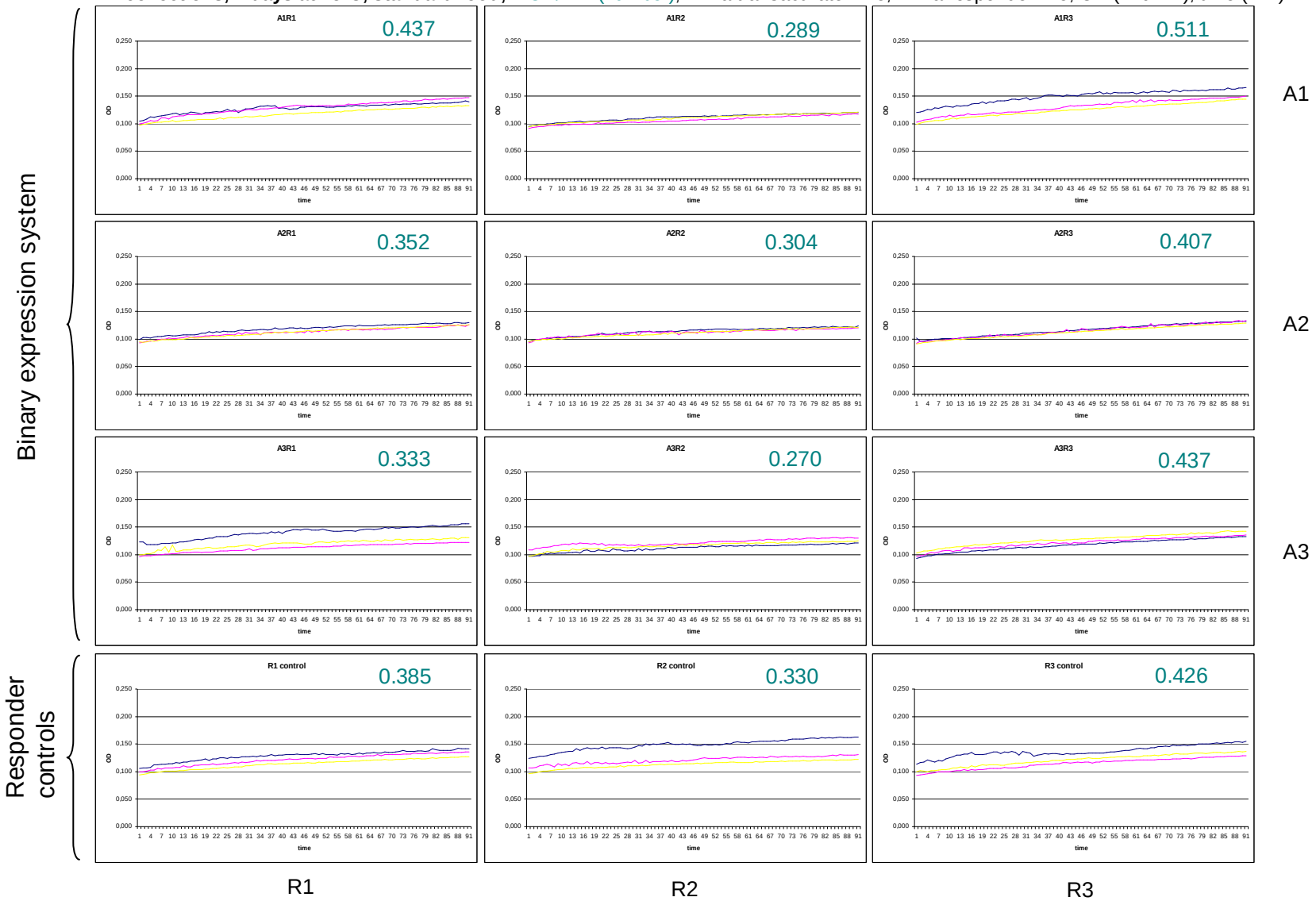
Drosophila melanogaster **scrtTA/*TRE*** *second measurement*

24 h collections, 7 days at 25ºC, food with tetracycline (100 µg/ml) in the dark,
mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



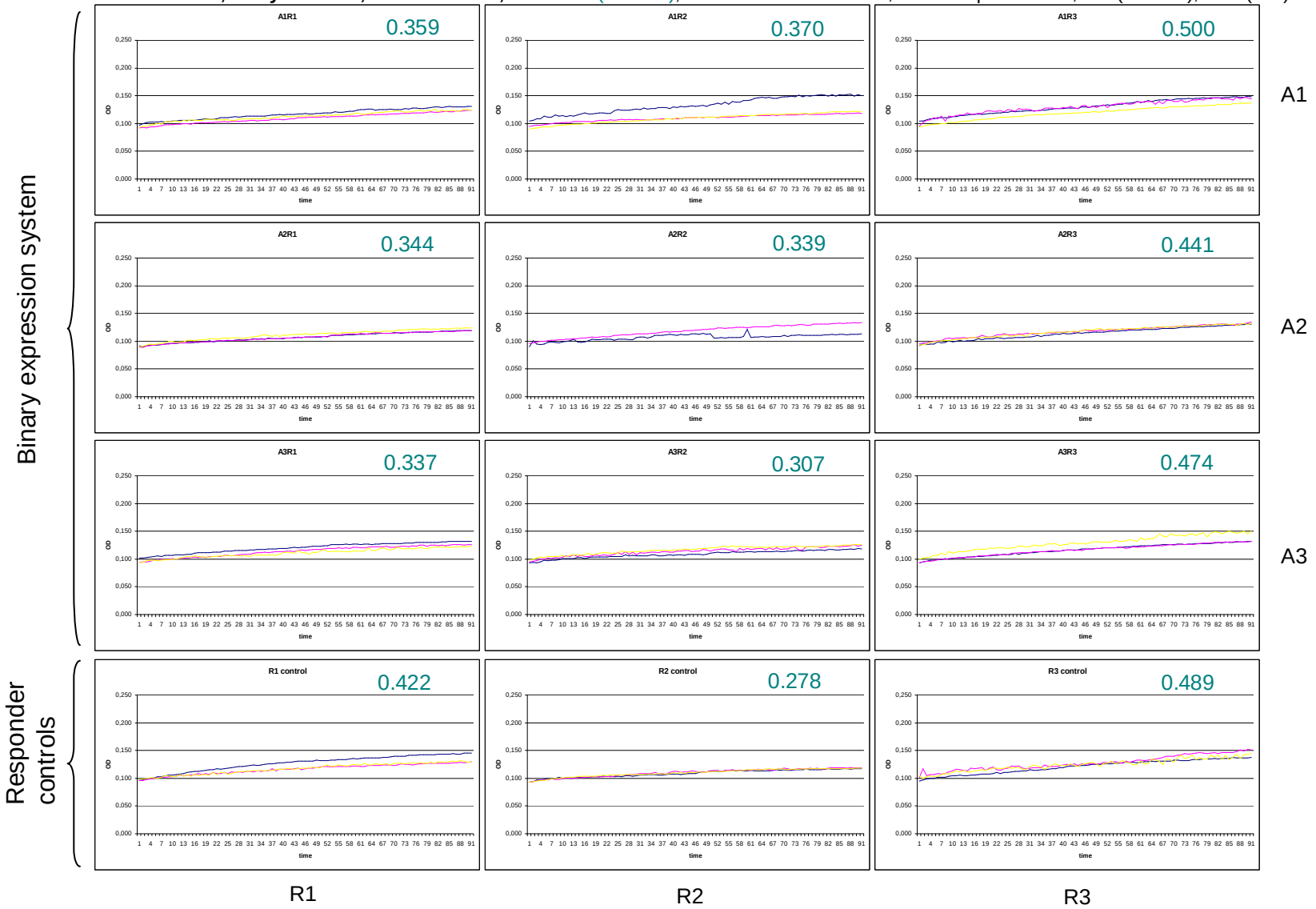
Drosophila melanogaster **scrtTA*ITRE*** *first measurement*

24 h collections, 7 days at 25ºC, standard food, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



Drosophila melanogaster **scrtTA/*TRE*** *second measurement*

24 h collections, 7 days at 25ºC, standard food, mOD/min (number); **A** = a transactivator line, **R** = a responder line, OD (410 nm), time (min)



ERKLÄRUNG

Hiermit versichere ich, die vorliegende Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt zu haben.

Ferner erkläre ich, dass ich weder an der Universität Bayreuth, noch an einer anderen Hochschule versucht habe, eine Dissertation einzureichen, oder mich einer Promotionsprüfung zu unterziehen.

Ivana Viktorinová

Bayreuth, Mai 2005