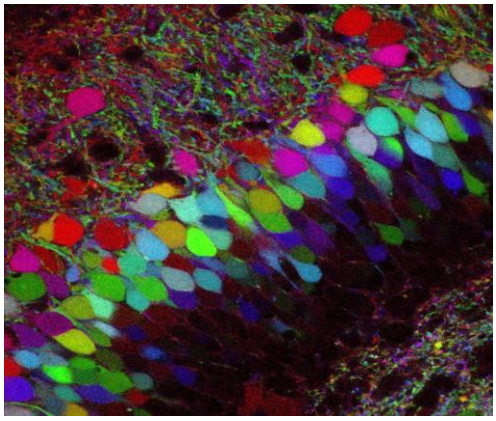
UNIVERSITY OF CRETE
DEPARTMENT OF BIOLOGY

"Η καθιέρωση ενός γενετικού εργαλείου για μωσαϊκή ανάλυση στο σκαθάρι *T. castaneum*"



Νευρώνες εκφράζουν το*brainbow* στο ΚΝΣ ενός ποντικιού

Ενήλικο *T. castaneum*

Μεταπτυχιακή εργασία της

Καλογεροπούλου Ελπινίκης

Επόπτης: **Μιχάλης Αβέρωφ**

ΗΡΑΚΛΕΙΟ 2010

Η εργασία εκπονήθηκε στο Ινστιτούτο Μοριακής βιολογίας και Βιοτεχνολογίας, τμήμα του Ινστιτούτου Τεχνολογίας και Έρευνας, στα πλαίσια των σπουδών του μεταπτυχιακού προγράμματος του Πανεπιστημίου Κρήτης 'Μοριακή Βιολογία και Βιοϊατρική'.

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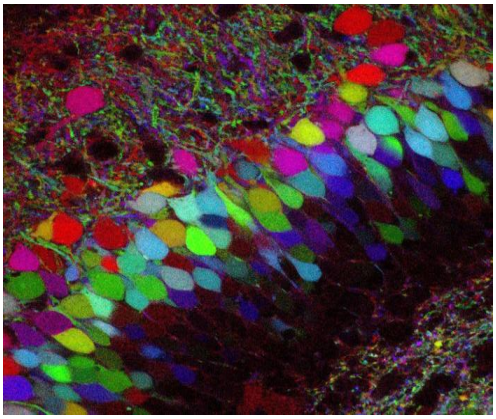
Εξεταστική επιτροπή: Μιχάλης Αβέρωφ, Χρήστος Δελιδάκης και Δημήτρης Τζαμαρίας

Εξώφυλλο : (αριστερά) Νευρώνες εκφράζουν το*brainbow* στο ΚΝΣ ενός ποντικιού (Livet *et al*, 2008), (δεξιά) Ενήλικο *T. Castaneum* (www.beetle.net)

UNIVERSITY OF CRETE
DEPARTMENT OF BIOLOGY

"Establishing a genetic tool for mosaic analysis in the red flour beetle *T. castaneum*"



Neurons expressing *Brainbow* in the CNS of mice

Red flour beetle *T. castaneum*

MSc project of

Kalogeropoulou Elpiniki

Supervised by **Michalis Averof**

HERAKLION 2010

Project was elaborated in the Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, within the terms of the Department of Biology's (University of Crete) MSc program 'Molecular biology and Biomedicine'.

Supervised by Michalis Averof, principal investigator in the Evolution of development and morphology lab, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas.

Project committee: Michalis Averof, Christos Delidakis and Dimitris Tzamarias

Cover images : (left) Neurons expressing *Brainbow* in the CNS of mice (Livet *et al*, 2008), (right) Red flour beetle *T. Castaneum* (www.beetle.net)

☙ *Acknowledgements* ❧

First of all, I would like to express my gratitude to Michalis Averof, my supervisor in this project work for giving me a place in his lab, for his help, guidance and patience throughout the project. It has always being a pleasure and a great interest ‘talking science’ with him.

Many thanks to Meriem Takarli for her daily help and showing me the do's and the don’ts when working with *T. castaneum*, Andrew Peel for his in situ and antibody staining guidance and the never-ending hours at the confocal microscope, Giannis Leivadaras for injecting so many eggs without any complaint, Johannes Schinko for the creation of the constructs injected, Nikos Konstantinides for the Merlin protocol and detailed explanation, primer designing and solving ‘the elusive mystery of the polymerase’, Harris Kontarakis for ‘molecular stuff’ and keeping my bench always occupied , Andrés Sarrazin for his hints and tips in live imaging,. All people working in the department that made my days in the lab easier and more cheerful.

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II. Abstract

In recent years, new genetic tools have been established that enhance our ability to study in detail gene function, neuronal networks and cell and organism architecture across a range of different species. In this MSc project I focus on establishing the genetic tool *brainbow* in the emerging arthropod model *Tribolium castaneum*. *Brainbow* is a construct carrying 4 different fluorescent proteins and variants of lox sites flanking each one of them. In a transgenic animal carrying both a *brainbow* and a *cre recombinase* expressing construct, the lox sites recombine stochastically. This results in clones of cells, genetically labelled with different fluorescent proteins. First introduced in mice by Livet *et al*. 2007, *brainbow* was targeted to the CNS. I have made the first attempt to target *brainbow* expression ubiquitously in *Tribolium castaneum*. If successful, cells should be marked with different fluorescing proteins, such that their progeny can be tracked *in vivo*, enabling us to investigate how the growth zone elongates and segments form in *Tribolium castaneum*.

I generated two different types of transgenic lines, some carrying a construct expressing *cre* recombinase (eight lines) and others carrying the *brainbow* construct (three lines). *cre* and brainbow lines were crossed with each other and then tested by PCR and imaging for efficient recombination of the constructs.

PCR experiments suggest that the cre/lox system is working in vivo in *Tribolium castaneum*. Recombination between the lox variants is happening. In addition, confocal microscopy revealed cherry protein labelled clones, resulting from recombination between one of the pairs of *lox* sites. However, the observed clones are few, rare and very small. Results show that the heat shock promoter used to mediate *cre* recombinase expression is leaky.

KEYWORDS: brainbow, *Tribolium castaneum*, cre / lox, transgenesis, mosaic analysis, multicolour imaging

III. Περίληψη

Τα τελευταία χρόνια, νέα γενετικά εργαλεία έχουν καθιερωθεί που μας δίνουν τη δυνατότητα να μελετήσουμε λεπτομερώς τη λειτουργία γονιδίων, νευρωνικά δίκτυα και την αρχιτεκτονική του κυττάρου σε μια σειρά διαφορετικών ειδών. Στην παρούσα μεταπτυχιακή μου εργασία εστιάζω στην καθιέρωση του γενετικού εργαλείου *brainbow* στο σκαθάρι *Tribolium castaneum*. Το *brainbow* είναι μία κατασκευή που φέρει 4 διαφορετικές φθορίζουσες πρωτεΐνες και παραλλαγές των περιοχών *lox,* τα οποία πλαισιώνουν την κάθεμιά απο αυτές. Σε ένα διαγενετικό ζώο που φέρει το *brainbow* αλλά συνάμα εκφράζει και τη *cre* ρεκομπινάση, οι περιοχές lox ανασυνδυάζονται τυχαία. Αυτό οδηγεί στη δημιουργία κλώνων κυττάρων, που σημαίνονται γενετικά με τις διαφορετικές φθορίζουσες πρωτεΐνες. Το brainbow εισήγαγε η Livet et al, το 2007 και εκφράζονταν επιλεκτικά στο ΚΝΣ των ποντικιών. Στην παρουσα εργασία έκανα την πρώτη προσπάθεια να στοχεύσω την ευρεία έκφραση του *brainbow* στο *Tribolium castaneum*. Εάν επιτυχής, τα κύτταρα πρέπει να μαρκαριστούν με τις διαφορετικές φθορίζουσες πρωτεΐνες, έτσι ώστε οι απόγονοί τους να μπορούν να ακολουθηθούν *in vivo,* επιτρέποντας σε μας να ερευνήσουμε πώς η ζώνη ανάπτυξης (growth zone) επιμηκύνεται και πώς τα μεταμερή δημιουργούνται στο *Tribolium castaneum*.

Παρήγαγα δύο διαφορετικούς τύπους διαγενετικών σκαθαριών, ο ένας φέρνει μία κατασκευή εκφράζοντας τη cre ρεκομπινάση (οκτώ strains συνολικά) και ο άλλος φέρνει την κατασκευή brainbow (τρία strains). Έπειτα απο διασταύρωση των διαγενετικών τύπων, *cre* και *brainbow,* εξετάστηκε με τη μέθοδο της PCR και την μικροσκοπία ο επιτυχής ανασυνδυασμός των κατασκευών.

Οι αντιδράσεις PCR υπέδειξαν ότι το σύστημα cre/lox λειτουργεί στο *Tribolium castaneum*. Ο ανασυνδυασμός μεταξύ των διάφορων περιοχών lox συμβαίνει. Επιπλέον, η ομοεστιακή μικροσκοπία (confocal) αποκάλυψε κλώνους κυττάρων που φθορίζανε στο κόκκινο χρώμα, ως αποτέλεσμα του ανασυνδυασμού μεταξύ ενός από τα ζευγάρια των περιοχών lox. Εντούτοις, οι παρατηρηθέντες κλώνοι είναι λίγοι, σπάνιοι και πολύ μικροί. Τα αποτελέσματα επίσης δείχνουν ότι ο heat shock promoter που χρησιμοποιείται για να επάγει την έκφραση της cre ρεκομπινάσης είναι leaky.

Λέξεις κλειδιά : brainbow, *Tribolium castaneum*, cre / lox, transgenesis, mosaic analysis, multicolour imaging

1. Introduction

In recent years, new genetic tools have been established that enhance our ability to study in detail gene function, neuronal networks, cell and organism architecture across a range of different species. The need for such new tools and more reliable techniques, as we dive into more and more demanding questions in research, is as important as ever.

This MSc project focused on establishing a newly introduced genetic tool to the red flour beetle *Tribolium castaneum*.

1.1 Cre/Lox expression system

The Cre/Lox system, largely used in mammalian models, was introduced in the 1980's (Sternberg and Hamilton 1981; Sauer and Henderson 1988). It is based on the ability of the P1 bacteriophage recombinase gene *cre* to effect recombination between pairs of lox sites. A lox site is a specific 34bp sequence, of the P1 bacteriophage, consisting of an 8 bp core where recombination takes effect. Recombination can take place either by DNA excision, inversion or interchromosomal recombination and can be determined by the orientation of the lox sites (*figure 1.1*). Such recombination can either activate or inactivate a gene of interest. Up to this day the system has been successfully applied to yeasts, plants, mammalian cell cultures, mice and *D. melanogaster*.

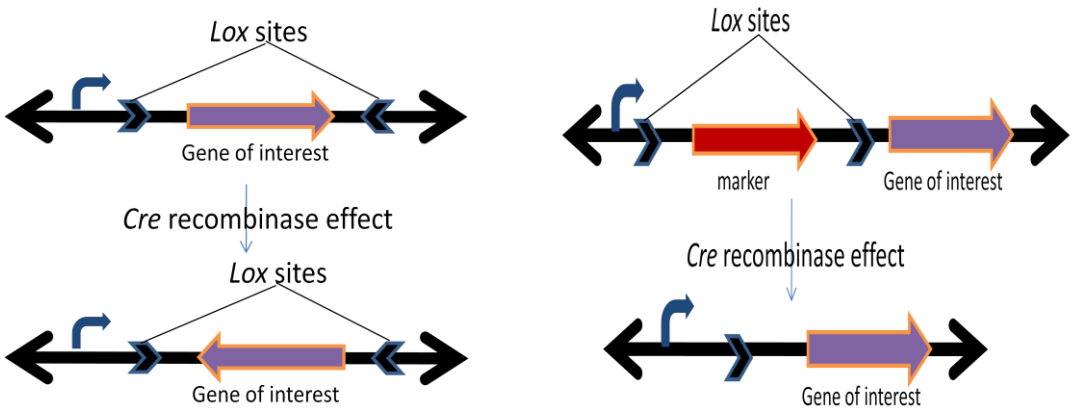

Figure 1.1Simplified schematic representation of inversion (left) and excision (right), in the cre/lox system, depending on the orientation of the *lox* sites.

In the genomes referred to above, the *cre* gene and *lox* sites are not native; hence they must be introduced by transgenic technology. *cre* and *lox* strains are developed independently, and then crossed. Depending on which strains are crossed, a scientist can implement a variety of *cre*-mediated

applications such as knockouts, chromosome aberrants, diet-induced mutants; hence having the opportunity to study a gene's effect in tissue- and developmental stage-specific ways. A gene of interest can be either activated or inactivated by directed deletion, inversion or interchromosomal recombination, depending on the orientation of the lox sites. Cre recombinase can be induced with chemical treatments, heat shock treatments or be restricted to specific tissues, depending on the promoter under which is being mediated. For example, if *cre* gene was under either a tamoxifen-induced promoter, a heat shock promoter or a promoter targeted in the muscles, it would be expressed in the presence of the chemical tamoxifen, when we have a significant change of temperature or specifically in the muscles, in retrospect.

1.2 *Brainbow* construct

The *Brainbow* construct was first introduced to the central nervous system of mice by Livet *et al*, 2007, mediated by the Cre recombinase – Lox sites interaction. It consisted of genes encoding four fluorescent proteins (red, yellow, orange, cyan) and variants of *lox* sites (loxN, lox2272, loxP) flanking each one of those proteins (figure 1.2). Once *cre* mediated recombination took place, different cells were marked in a variety of fluorescent colours since different proteins were activated in each cell, stochastically.

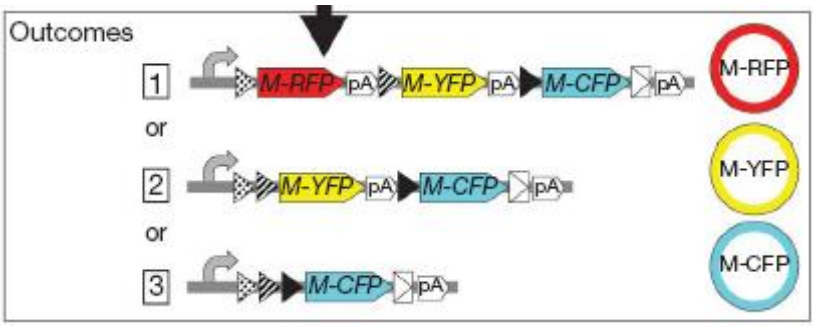

Figure 1.2 Schematic representation of *Brainbow* construct introduced to the CNS of mice and its outcomes after the interaction with *cre* recombinase. Image by Livet *et al*, 2007.

In total, up to 90 colours were distinguishable in the brain cells of these mice carrying *brainbow (figure 1.3)* since there were multiple insertions in the genome of the construct.

The system can be used to show how cells interweave, provides a way to distinguish adjacent neurons and visualize other cellular interactions, and allows researchers to map glial territories and follow glial cells and neurons over time *in vivo* (Livet *et al*, 2007).

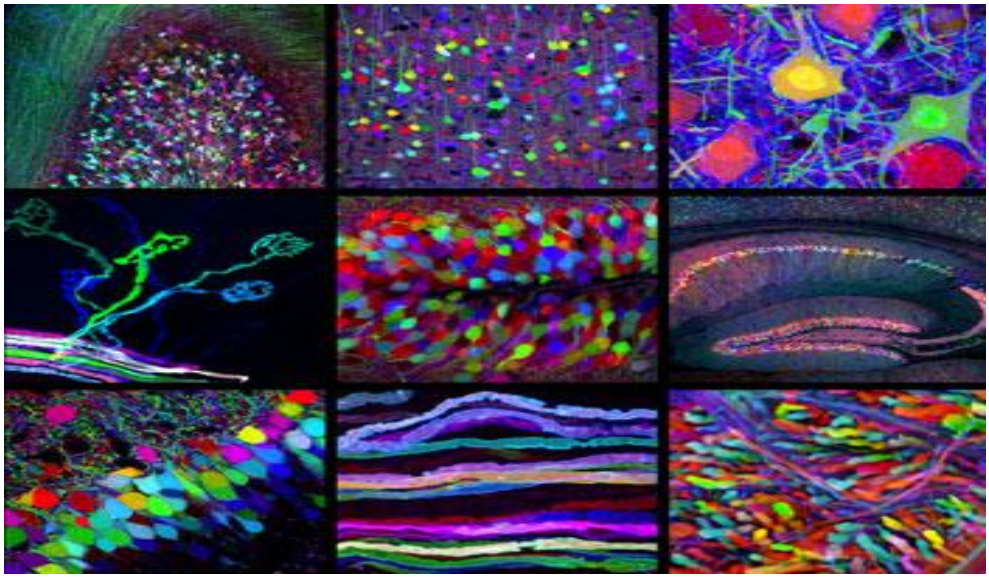

Figure 1.3 Neuronal cells of mice CNS genetically labelled by the *Brainbow - cre* mediated system. Images by Livet *et al, 2007*

1.2.1 Brainbow in other models

Brainbow has already been introduced successfully to zebrafish , sp. *Danio rerio*, by Albert Pan from Harvard University (figures 1.4 *a* and 1.4 *b* , data not published yet). Moreover, there have been attempts to introduce *brainbow* in the tracheal system of *Drosophila melanogaster* by Luchnig and Förster from University of Zurich, (figure 1.6, data not published yet).

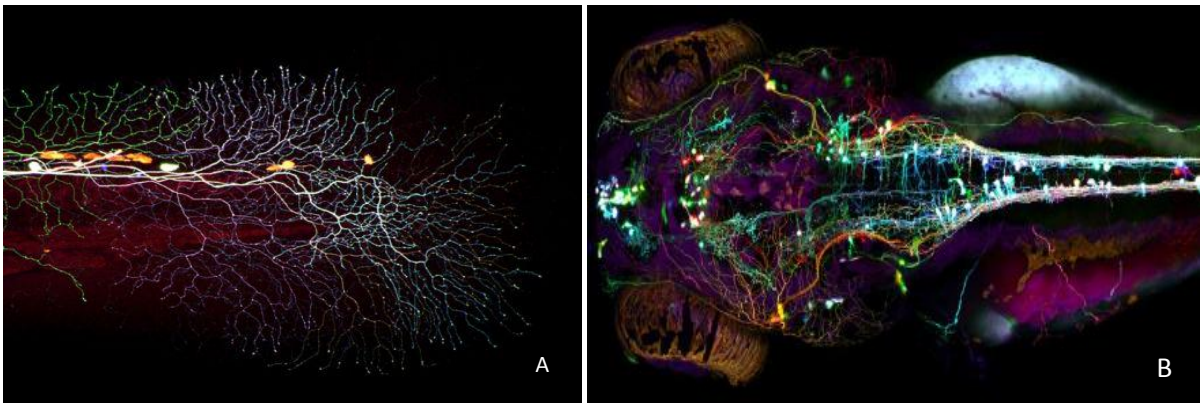

Figure 1.4 a) Sensory axons covering the tail of a 3- day-old zebrafish expressing *brainbow*. b) 5- day-old zebrafish expressing *brainbow*. Confocal microscopy. Images by Albert Pan, Harvard University, 2009 (Olympus BioScapes Digital Imaging competition).

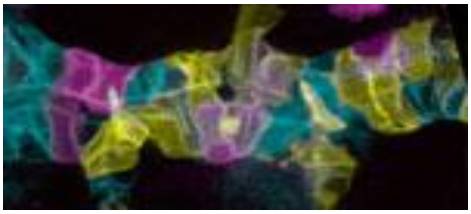

Figure 1.5 Tracheal tube in Drosophila melanogaster expressing *brainbow*. Confocal microscopy. Image courtesy of Dominique Förster and Stefan Luschnig, Kolymbari Drosophila meeting 2010.

1.2.2 Possible uses of brainbow under the cre/lox system concerning our area of interest

In addition to the uses of *brainbow* referred to above, by Livet *et al*, 2007, this construct could give us the opportunity to examine in vivo cell proliferation in the growth zone, an area we are interested in. By clonal analysis and following cells over time in vivo, it can be used for experiments concerning segmentation and axis elongation. By expressing *brainbow* to the cells that possibly give birth to the elongating growth zone or to a subset of cells in the growth zone, cells will be marked with different fluorescing protein and we could then track their progeny. We could then be able to investigate how growth zone elongates and the formation of segments.

1.3 The use of *Tribolium castaneum* as a model

The red flour beetle *Tribolium castaneum* is lately considered as an emerging arthropod model for studying the evolution of development due to its advantages, such as cheap maintenance, a three weeks generation time and various genetic tools. Possible RNA interference experiments at any developmental stage, small and sequenced genome since 2008, enhancer gene traps, genetic maps for visible and molecular markers, chromosomal rearrangements that balance lethal mutations, EMS and transposons mutagenesis, make it a valuable model for both reverse and forward genetics. Undergoing a more ancestral early development mode (short germ embryogenesis) to the specialised development mode of the well established arthropod model *Drosophila melanogaster* (long germ embryogenesis), it is a valuable model for comparative studies. It has been used for biological questions regarding segmentation in a growing germ band, head development, leg development etc. (Brown *et al.,* 2003 and 2008; Klinger 2004).

Prior to this MSc project, the Cre/lox system had not been tested and *brainbow* had not yet been introduced to *Tribolium castaneum.*

1.5 Aims of this Msc project

During this project I aimed to introduce the *brainbow* construct to the red flour beetle *Tribolium castaneum*. For this reason two transgenic lines were created, one carrying a construct expressing *cre* recombinase and another carrying *brainbow*. Beetles were screened for successful transgenesis and expression of the genes of interest, and tested with molecular and imaging procedures for the efficient recombination of the constructs.

Finally, I will discuss my findings, speculate the reasons the system works or not efficiently and discuss the future prospects arising from these results.

2. Materials and Methods

2.1 Model organism: culturing and stock keeping

The model organism used throughout this project is the red flour beetle *T. castaneum*, a holometabolous insect that belongs to the Coleoptera order of insects. It grows on full grain flour, containing yeast and the antibiotic, fumidil, at temperatures from 20 to 40 °C. In the lab, beetles were normally grown at a temperature of 32 °C resulting to a life cycle of 22 days. Temperature influences developmental speed to a great extent as shown in *table 2.1*.

Table 2. 1 Tribolium life parameter table (*Data from Sokoloff, 1972*)

Tempearture (°C)	Embryonic development (days)	Larval development (days)	Metamorphosis (days)	Total development time (days)
37.5	2.6	13.7	3.9	20
32.5	2.9	14.6	4.6	22
30	3.6	17.2	5.5	27
25	6.8	31.2	10.2	48
22.5	9.3	51	13.4	74

Under normal conditions, as followed here, Tribolium has 6 larval instars. Females mature on the seventh day after eclosion and males on the second when kept at a temperature of 25°C. For egg collections, crosses were placed on white flour overnight without the presence of yeast, making it possible to separate the eggs from the adults with a laboratory sieve of 250μm aperture.

2.2 Constructs injected for the creation of the transgenic lines

For the *cre/lox* system to work, the *cre recombinase* gene and the *lox sites* must be separately introduced to two different lines of the beetles. Once the transgenic lines are created, they can be then crossed with each other and the recombination can take effect. The two constructs were stably integrated into the genome of the *T.castaneum* by transposition, using *piggy Bac* vectors. In both constructs gene expression is driven by cis-regulatory elements derived from Tribolium castaneum. This is required for efficient transgene expression (Schinko *et al*, 2010). Constructs were made by Johannes Schinko.

2.2.1 Cre recombinase construct

Cre construct (entitled Cre3 in our lab stocks and referred to, in this project henceforth, as Cre) was created by cloning a 2.2 kb fragment cut with restriction enzyme AscI from pSLfa (hspCre recombinase – 3’ UTR) vector into a piggybac(3xP3-EGFP) vetor, cut with AscI. Thus, Cre is

expressed under the control of a Tribolium heat inducible promoter and the marker for the transgenic animals is the green fluorescent protein under the control of the artificial *3xP3* promoter (Berghammer *et al*, 1999), expressing the fluorescent protein in the eyes (*figure 2.1*).

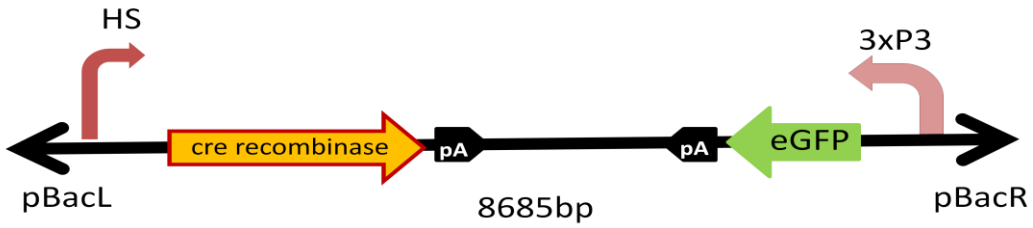

Figure 2.1 Schematic representation of *Cre* recombinase construct

2.2.2 Brainbow construct

Brainbow construct (entitled BB7 in our lab stocks and referred to, in this project henceforth, as BB) was created by cloning an 8 kb fragment cut with restriction enzyme AscI from pSLfa(EFA – Brainbow 1.1 minus Kusabira) vector into a piggyBac(3xP3-DsRed) vector, cut with AscI. Thus, Brainbow is expressed under the control of a Tribolium ubiquitous promoter (EFA) and the transgenesis marker is red fluorescent protein (dsRED) under the control of the artificial *3xP3* promoter (Berghammer *et al*, 1999), expressing the fluorescent protein in the eyes (*figure 2.1*).

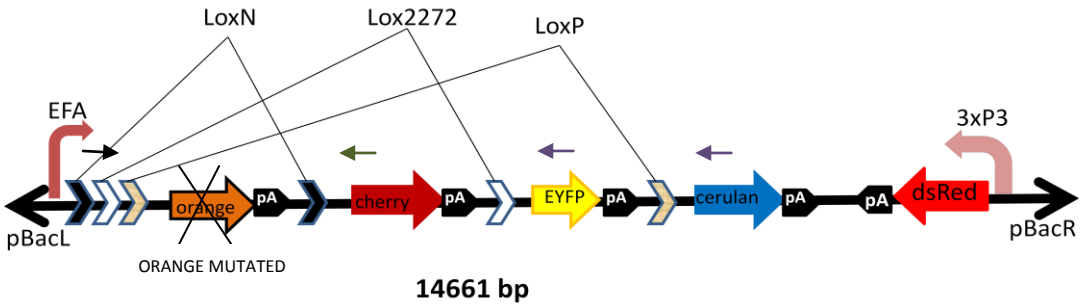

Figure 2.2 Schematic representation of *Brainbow construct*

2.2.3 Expected results of recombination

Once recombination is successful, the results expected from our *brainbow* construct are as shown in *figure 2.3.* Lack of recombination will not 'mark' cells, since the orange fluorescent protein is mutated in our construct.

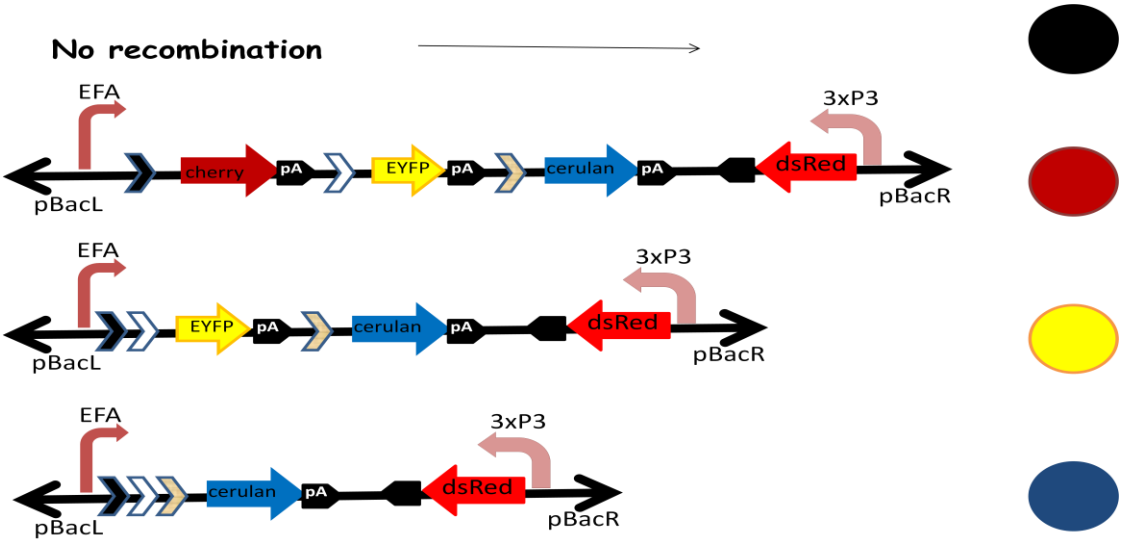

Figure 2.3 Schematic representation of recombination results expected between the two constructs

2.3 Injection process

Injections were carried out according to Berghammer *et al*, 2009. Dechorionated eggs, collected from an hour collection on white flour and left to grow for an additional hour, were aligned on a slide and injected with purified transposon vector and helper plasmids into the posterior pole of precellular embryos.

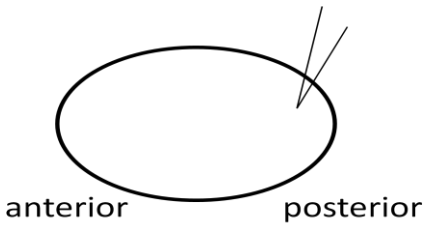

Figure 2.4 Schematic representation of the injected area

Specifically, the injection mix we used for *cre recombinase lines* consisted of 300 ng/µl piggy Bac helper plasmid, 700 ng/µl CRE plasmid, injection buffer and Phenol red. Whereas for *brainbow lines*, injection mix consisted of 300 ng/µl piggy Bac helper plasmid, 300ng/ µl BB plasmid, injection buffer and Phenol red. Injection buffer was prepared as standard for Drosophila injections ('Spradling injection buffer' *CSH Protocols*, 2008). Diagnostic digestions with AscI ensured that the right BB and CRE constructs were injected.

2.4 Screening and maintaining the transgenic lines

After being injected, eggs where incubated and looked after closely until hatching (3 days). Water was added twice per day in order to keep the humidity levels appropriate for a successful hatching. Hatchlings where carefully transferred, immediately after they had hatched, into whole wheat flour with the use of a small brush. Once in pupa stage (G0), they were separated according to

their sex in order to obtain the female beetles virgins (*figure 2.5*). Each pupa was then crossed with wild type tribolium and when their progeny (G1) reached the pupa stage (30 days approximately after the cross) they were screened under a Fluorescence Stereomicroscope (Leica MZ 16 F), for the expression of the 3xp3 marker in their eyes. The positive pupae (G1) for the 3xp3 marker were put afterwards in a breeding program as shown below in order to establish and maintain the transgenic lines (*figure 2.6*). Once every month, wild type beetles that emerged through the crosses were removed from the transgenic lines.

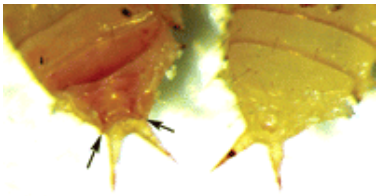

Figure 2.5 Pupae sex can be distinguished by the sexual dimorphism in the posterior tips of the genitalia (arrows), which are absent in males (*right*) but present in females (left). Image by Brown *et al*, 2008.

Figure 2.6 Schematic representation of the 'breeding program' followed to establish the transgenic lines

2.5 Heat shock treatment (inducing the expression of *cre* recombinase)

In order to induce the expression of *cre* and check if recombination takes place, virgins from a *cre* line were mated to virgins carrying *brainbow* (10 to 15 beetles per line). The cross was left on whole wheat flour for 5 days at 32 °C. Female beetles need to be fed on whole wheat flour before laying eggs and their ovaries are mature 3.5 days after eclosion at 32 °C (The beetle book, 2009). The cross was then transferred to white flour and left at 32 °C for an overnight egg laying. Egg collection took place the day after and the eggs were left to develop for another 6 hours. Heat shock treatment lasted for 10 minutes at a temperature of 47 °C, as previously established while introducing the binary system UAS/GAL4 in *Tribolium castaneum* (Schinko *et al*, 2010). Embryos treated were developed for 6 hours to 22 hours. Fixation, mounting and observation under a confocal microscope or live observation under a fluorescent steremicrooscope took place the day after heat shock treatment. The embryos observed were from a variety of developmental stages, starting from a development of 24 hours at 32°C. The fluorescent proteins need a few hours to be expressed and detected (Schinko *et al*, 2010), and this time of development also ensures that zygotic expression has already commenced.

In the beginning, heat shock treatment experiments were also applied at a temperature of 37 °C without noticing any reduced lethality or difference in the number of clones observed, with the temperature of 47 °C, which was the one used henceforth.

While using the *cre* line CRE-16 established by the lab of Gregor Bucher, the eggs were put in a waterbath of 47°C for 15 minutes and all other stages of the experiment took place strictly under 25 °C. In order to observe the same developmental stages as with the embryos breed at a temperature of 32°C, all steps of the experiment took twice the time.

2.6 Antibody staining for EGFP and Dsred proteins

Antibody stainings were carried out according to the established protocol of Patel, 1994. Primary antibodies used were anti-DsRed rabbit polyclonal antibody (Clontech p/n 632496) and anti-GFP mouse specific antibody (Invitrogen c/n 33-2600) (1:300 using the stocks available in our lab). Secondary antibodies used were anti-mouseIgg488 and anti-rabbitIggRCy3 (1:100 using the stocks available in our lab). The antibody of GFP cross reacts with YFP and CFP fluorescent proteins (see antibodies at www.clontech.com). CFP and YFP are derivatives of GFP with a few point mutations only.

2.7 Primers designed for PCR

In order to test if recombination takes effect between the first pair of *loxN* sites two primers were designed:

BB7loxF FORWARD: 5’ – TGTTGCTAATGGGGTGGTTT - 3’, Tm = 60.2 °C
BrMloxRev2 REVERSE no1: 5’- CATGGTGGCGGATGCTAC-3’, Tm = 61.1 °C

For testing whether recombination takes place between the rest lox pairs (*loxP and lox2272*), another reverse primer had to be designed:

BB7Rrec2 REVERSE no2: 5’-ATGAACTTCAGGGTCAGCTTG-3’, Tm = 62 °C

The forward primer BB7loxF (black arrow in *figure 2.2*) initiates the reaction before the first lox site of the pair *loxN* and at the end of the EFA promoter. The reverse primer BrMloxRev2 (green arrow in *figure 2.2*) initiates the reaction at the beginning of *cherry* fluorescent protein gene and after the second lox site of the variant *loxN* pair. Whereas, the second reverse primer BB7Rrec2 (purple arrows in *figure 2.2*) initiates the reaction at two possible sites, inside the YFP gene and inside the CFP gene. A standard PCR program was used as shown below:

PCR program

Initial Denaturation	95 °C		2 min	
Denaturation		95 °C	30 sec	
Annealing		65 °C	30 sec	20 cycles
Elongation*		72 °C	2 min	from 66 °C to 59 °C
Denaturation		95 °C	30 sec	
Annealing		55 °C	30 sec	20 cycles or 35 cycles
Elongation*		72 °C	2 min	
Final Elongation	72 °C		7 min	
HOLD		*12 °C*		

*For bands longer than 2kb elongation time was extended to 3 minutes.

2.8 DNA extraction from beetles

DNA extractions from adult beetles were carried out according to the standard protocol of DNA extraction for *Drosophila melanogaster.* Protocol shown below:

- 12 tribolia at -80°C
- Add 200µl of Buffer A* and grind
- Add 200µl of Buffer A* and grind until cuticles remain
- Incubate at 65°C for 30 min
- Add 800 µl of LiCl/KAc* and incubate on ice for 10 min
- Spin down for 15 min
- Remove supernatant to new tube and spin again
- Add 600 µl isopropanol, mix and spin down for 15 min
- Aspirate away supernatant and wash pellet with ethanol
- Let it dry
- Resuspend in TE
- Stock at -20°C

* *Buffer A*
100mM Tris-HCl, ph= 7.5
100mM EDTA
100mM NaCl
0.5% SDS
LiCl/KAc
1 part 5M KAc : 2.5 parts 6M LiCl

2.9 Other strains used

I also crossed one of our BB lines with a CRE line established by the lab of Gregor Bucher. *Cre* line number 16 has an alternative marker system (VW) based on rescue of the white-eye mutation in the eye pigmentation gene *vermilion.* However, *cre* recombinase is expressed under a heat shock promoter the same way as in our strains.

3. Results

3.1 Injections and survival rates

In total 5 rounds of injections took place, each round referring to 3 days of injections. While establishing *cre* transgenic lines 2550 eggs in total were injected and for the *brainbow* lines 1720 eggs. From these injections 478 and 641 larvae survived, respectively. Survival rates, from the first round of injections to the last improved from 5.4% to 42.5%, due in part to increasing the humidity level, decreasing the number of eggs aligned in each slide and making accurate collections and timely harvests of the larvae hatched from the slide to whole grain flour. See *tables 3.1* and *3.2* that follow.

Table 3.1 Number of eggs injected, larvae hatched and survival rates while establishing the *cre* transgenic lines

CRE	eggs injected	larvae hatced	survival rate
1st round	1000	54	5.4%
2nd round	1200	283	23.5%
3rd round	350	141	40%

Table 3.2 Number of eggs injected, larvae hatched and survival rates while establishing the *brainbow* transgenic lines

BrainBow	eggs injected	larvae hatced	survival rate
1st round	920	391	42.5%
2nd round	800	250	31%

3.2 Screening results and transgenic lines

From a total of 775 crosses of the survived injected beetles mated with wild type beetles, 11 successful transformants (G0) were found; a transformant rate of 2.4 % for the *cre* construct and almost 1% for the *brainbow* construct (*table 3.3 and 3.4*). Screening lasted overal 5 months and all of the crosses' progenies were screened under the fluorescent stereoscope for the expression of the 3xp3 marker in their eyes (*figure 3.1*).

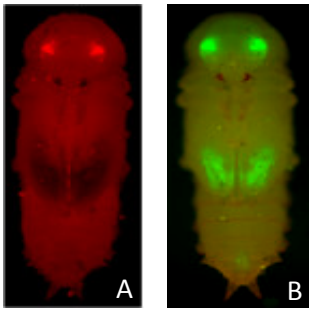

Figure 3.1 Expression of the 3xp3 marker in the progeny of the injected beetles. (A) *Brainbow* transformed beetles. (B) *Cre* transformed beetles.

Table 3.3 Number of crosses and transgenesis rates of the *cre* transgenic lines

CRE	crosses (x wt)	progeny screened	transformant Go
1st round	41	All crosses for at least 3 weekly collections of progeny	1
2nd round	180	All crosses for at least 3 weekly collections of progeny	6
3rd round	116	All crosses for at least 3 weekly collections of progeny	1
Total	337		8 (2.4%)

Table 3.4 Number of Crosses and transgenesis rates of the *brainbow* Lines

BrainBow	crosses (x wt)	progeny screened	transformant Go
1st round	247	All crosses for at least 3 weekly collections of progeny	3
2nd round	191	All crosses for at least 3 weekly collections of progeny	NO
Total	438		3 (≈ 1%)

The 8 transgenic lines that were established carrying the *cre* recombinase construct are entitled CRE-Voulis, CRE-Kinovio49, CRE-104, CRE-A, CRE-B, CRE-C, CRE-A' and CRE-B'. The 3 lines carrying the *brainbow* construct are entitled BB-178b, BB-215 and BB-206.

3.3 Checking whether recombination takes place by means of PCR

With the use of the first set of primers BB7loxF and BrMloxRev2, referred in the methods and materials section, we were able to investigate if recombination takes place between the first pair of lox sites (*loxN*). Sites with no recombination events whatsoever, should give us an amplified band of 1100bp, using the first set of primers. This is expected of purified plasmids carrying the *brainbow*, *brainbow* transgenic beetles and double transgenic beetles (progeny from BB beetles mated with CRE beetles) without a heat shock treatment. If recombination between the first pair of lox sites is successful after the heat shock treatment, then a band of 295bp from the double transgenic beetles is expected.

Our results while using CRE-Voulis crossed with BB lines BB-215 or BB-178b, as depicted in *figure 3.2*, partly confirmed our expectations.

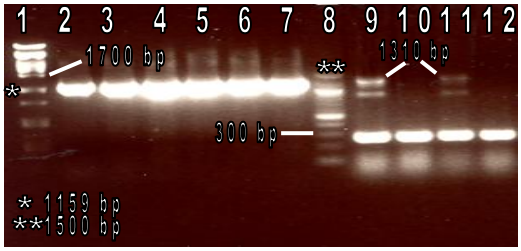

Figure 3.2 2% Agarose gel loaded with PCR product using the first set of primers, BB7loxF and BrMloxRev2. Lane 1: λ/PstI ladder. Lanes 2 and 3: Product from the DNA extract from beetles carrying *brainbow*. Lanes 4-7: Product from different concentrations of purified plasmids carrying the *brainbow* construct. Lane 8: 100bp ladder. Lane 9: Product from the DNA extract from double transgenic beetles, CRE-Voulis x BB-178b, without heat shock treatment. Lane 10: Product from the DNA extract from double transgenic beetles, CRE-Voulis x BB-178b, with heat shock treatment. Lane 11: Product from the DNA extract from double transgenic beetles, CRE-Voulis x BB-215, without heat shock treatment. Lane 12: Product from the DNA extract from double transgenic beetles, CRE-Voulis x BB-215, with heat shock treatment.

However, recombination takes place in the beetles that didn't undergo a heat shock treatment (see in *figure 3.2* lanes 9 and 11), which suggests that our heat inducible promoter is leaky. A comparison between the lines 9 and 10, 11 and 12, suggests that the heat shock treatment increases recombination, at least for the first set of lox sites (*loxN* variant).

Using the same forward primer and the second reverse primer BB7Rrec2, referred in the methods and materials section, we were able to investigate whether recombination takes place between *lox2272* and *loxP* sites. Two bands at 450bp and 1590bp are expected after recombination at *lox2272* sites and a band at 497 is expected after recombination at *loxP* sites. Although a band of 1590bp was not noticeable and an unexpected band of 1488bp in lane 4 was observed, the rest of our results agreed with what was expected (see lanes 6 and 7 in *figure 3.3*). For the experiment showed in *figure 3.3* the alternative cre line CRE16 was used. *cre* recombinase showed heat-independent expression in this line too, hence no difference was noticed in results from the beetles before and after the heat shock treatment.

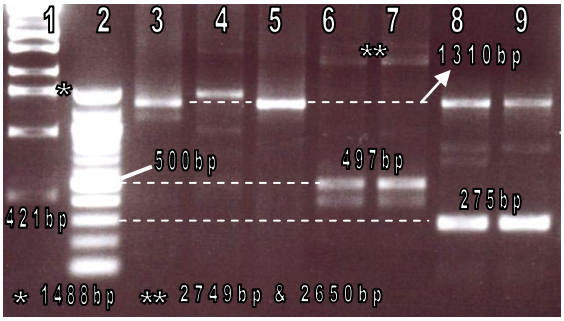

Figure 3.3 1.5% Agarose gel loaded with PCR product using all primers desinged, BB7loxF, BB7Rrec2 and BrMloxRev2. Lane 1: λ/styI ladder. Lane 2: 100bp ladder. Lanes 3 and 4: Product from the purified plasmid DNA extract with the BrMloxRev2 and BB7Rrec2 reverse primers, respectively. Lane 5: Product from the DNA extract from beetles carrying the *brainbow* with the BrMloxRev2 reverse primer. Lanes 6 and 7: Product from double transgenic beetles, cross CRE-16 x BB-215 with the BB7Rrec2 reverse primer. Lane 8 and 9: Product from double transgenic beetles, cross CRE-16 x BB-215 with the BrMloxRev2 reverse primer.

3.4 Live observation of embryos under a fluorescent stereomicroscope

Dechorionated embryos were observed live under a fluorescent stereomicroscope in the filter sets available (GFP, DsRed, CFP, and YFP). Throughout this project 4 cre lines were observed during embryonic development with or without heat shock treatment; CRE-Voulis *cre* line mated with BB-178b and BB-215 *brainbow* lines, CRE-A *cre* line mated with BB-178b brainbow line, CRE-B *cre* line mated with BB-206 *brainbow* line and *cre* line CRE-16 from Gregor's Bucher lab mated with our BB-215 *brainbow* line. Images shown below are of late stages. We focused on later stages since there was nothing observed in early embryonic stages before 18 hours of development. What is more, the later the stage the more chance we had to observe bigger clones.

3.4.1 The '3xp3 promoter' issue

The 3xp3 artificial promoter element, used here to drive expression of fluorescent proteins in the eyes of the successful transformants, was designed to bind three PAX 6 homodimers (Sheng et al. 1997). As has been observed before, the artificial 3xp3 promoter drives expression of the marker not only to the eyes but also to the CNS, peripheral nervous system, hindgut and the anal plates (Horn *et al*, 2000 and Berghammer *et al*, 2008). While experimenting with our cre lines, we constantly observed GFP in the central and peripheral nervous system, and with our brainbow lines we observed DsRed in the CNS, especially in the head lobes (*figure* 3.4). Patterns of fluorescent cells, resembling the ones depicted in figure 3.4, in later images shown in this project are neural cells from the CNS and PNS 'marked' by the 3xp3 promoter element, rather than clones from the expression of the *brainbow* construct.

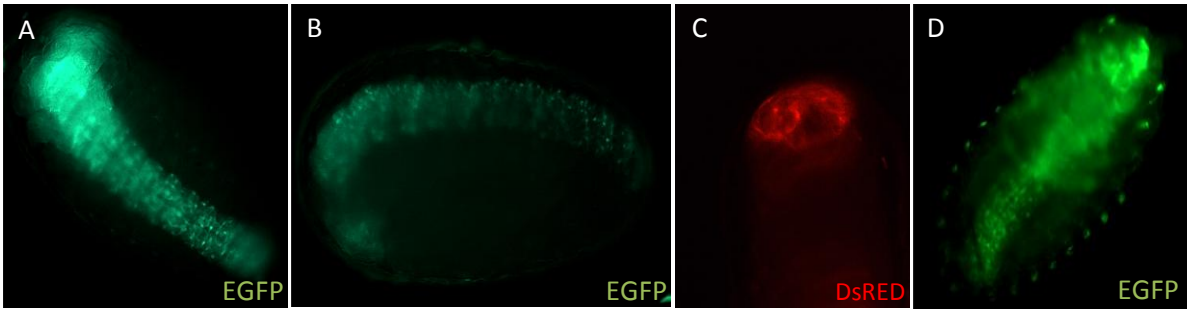

Figure 3.4 GFP and Dsred expression in the CNS and PNS driven by the 3xp3 artificial promoter. (A, B, D) Ventral, lateral and dorsal views of transgenic embryos from CRE-Voulis and CRE-A lines. Image D taken from an Olympus fluorescence microscope. (C) Dorsal view of transgenic embryo from 215 *brainbow* line. Embryos imaged on a fluorescence stereomicroscope with GFP, DsRed, YFP and CFP filters.

3.4.2 Embryos observed under the stereomicroscope without heatshock treatment

Untreated embryos from our crosses were observed at all embryonic stages. We spotted nothing more than the fluorescence from our transformation markers while using all the fluorescence filters (*figure 3.5*). Overall, approximately 100 to 150 embryos from each of the 3 cre lines mentioned above, mated with the brainbow ones (BB-215, BB-206 and BB-178b), were checked without a heat

shock treatment; half of these embryos were carrying the *cre* recombinase gene and about ¼ of them carried both CRE and BB constructs.

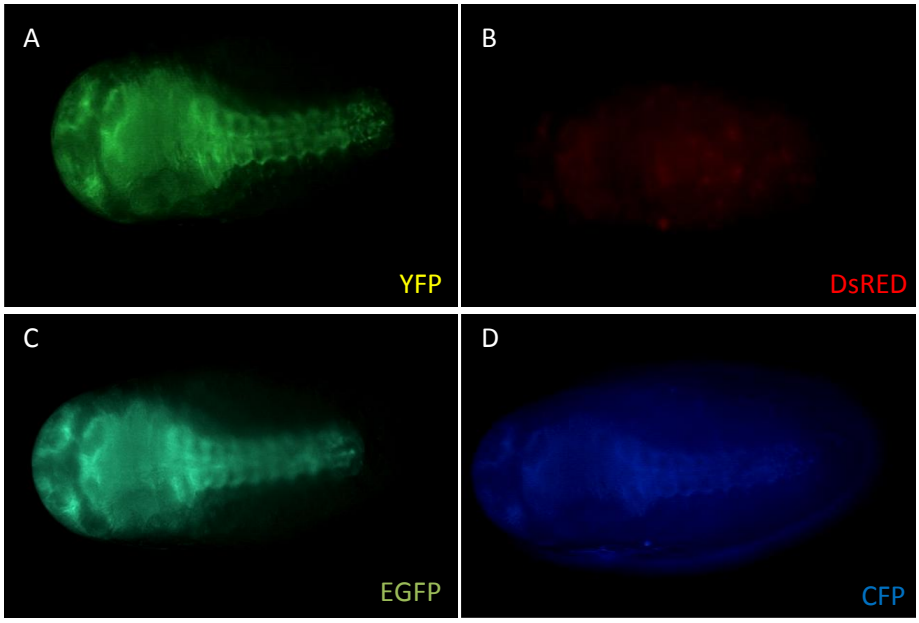

Figure 3.5 Ventral view of a transgenic untreated embryo from the cross: CRE-Voulis x BB-178b. Embryo imaged on identical magnification on a fluorescent stereomicroscope with GFP, DsRed, YFP and CFP filters.

3.4.3 Embryos observed under the stereomicroscope, after a heat shock treatment

Treated embryos, at a temperature of 47 °C, were observed at all embryonic stages. We observed, as with the untreated embryos, the fluorescence from our transformation markers in all embryos.

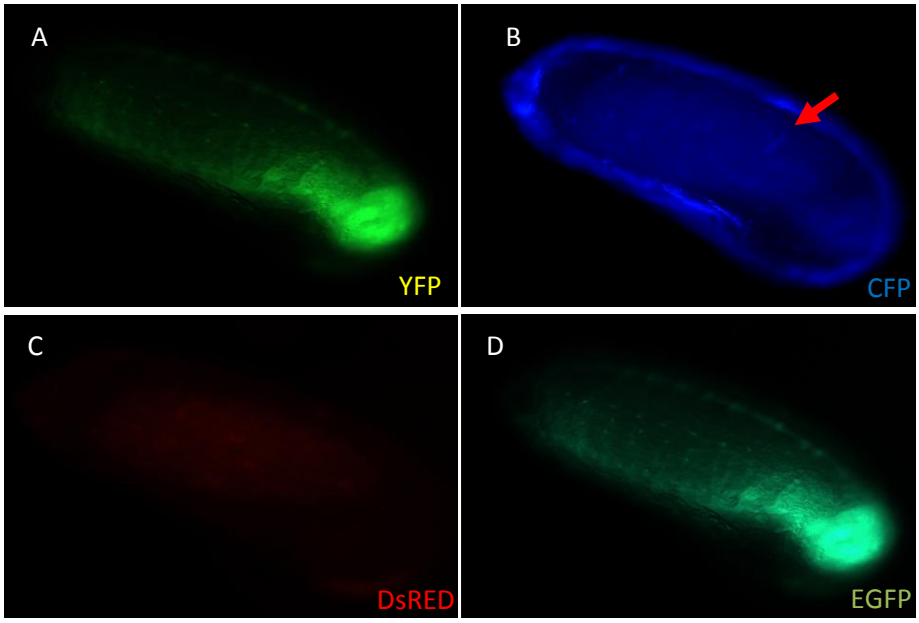

Figure 3.6.1 Lateral view of a transgenic embryo from the cross: CRE-Voulis x BB-215, 20 hours after heat shock treatment. Arrow shows a potential asymmetrical clone. Embryo imaged on identical magnification on a fluorescent stereomicroscope with GFP, DsRed, YFP and CFP Filters.

However, there were a few rare cases of small asymmetric patches of cells fluorescing in the CFP and DsRed filters (*figure 3.6.1 and 3.6.2*). No clones were spotted with the YFP filter, other than the overlapping emission of the GFP marker. Overall, approximately 100 to 150 embryos from each of the 3 *cre* lines mentioned above, mated with the brainbow ones(BB-215, BB-206 and BB-178b), were checked after a heat shock treatment; half of these embryos were carrying the *cre* recombinase gene and about ¼ of them carried both CRE and BB constructs. 3 of them appeared to have potential clones from the crosses CRE-A x BB-178b and CRE-Voulis x BB-215.

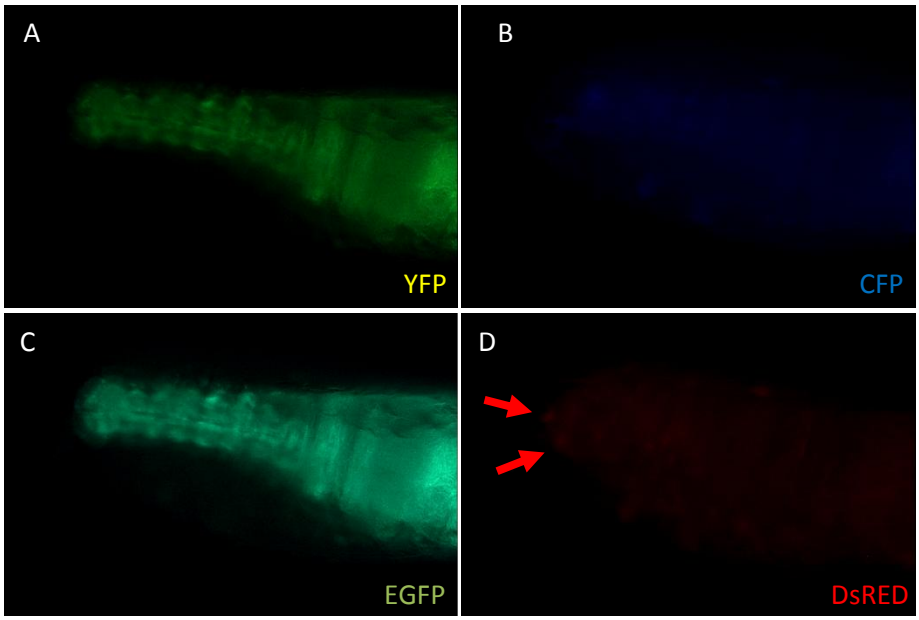

Figure 3.6.2 Ventral view of a transgenic embryo from the cross: CRE-A x BB-178b after 20 hours of heat shock treatment. Arrow show potential asymmetrical clones; rest of the red spots in image D are the fluorescent yolk in DsRed. Embryo imaged on identical magnification on a fluorescent stereomicroscope with GFP, DsRed, YFP and CFP filters.

3.4.4 Embryos observed while using a cre line established by Gregor Bucher

The expression of the 3xp3 marker, mainly in the CNS and PNS of the embryos that interfered with our observations, the low percentage of potential clones spotted and the apparent leakiness of the heat shock promoter, under which *cre* is being expressed, prompted us to use an alternative *cre* line. *Cre* line number 16, established by Gregor Bucher's lab, has a non-fluorescent transformation marker (vermilion white) but, as previously shown in figure 3.3, a leaky heat shock promoter. The absence of the GFP fluorescent marker helped us in our investigation of potential clones; VW marker from the *cre* line never interfered with our observations and the 3xp3-DsRed from the *brainbow* line was fainter than the GFP one, and showed delayed expression, giving us the time to search for potential clones in many embryonic stages.

Overall, approximately 210 embryos from the line CRE-16, mated with the brainbow line BB-215, were checked without a heat shock treatment; more than half of them were carrying the *cre* recombinase gene and 48 of them carried both transgenes. No clones were observed (*figure 3.7.1*).

In parallel, approximately 240 embryos from the cre line 16, mated with the brainbow line 215, were checked more than 18 hours after a heat shock treatment at 47°C; more than half of them were carrying the *cre* recombinase gene and 72 of them were carrying both transgenes. Overall, 2 potential clones were observed (*figure 3.7.2*). However, we could not follow any of them during subsequent stages.

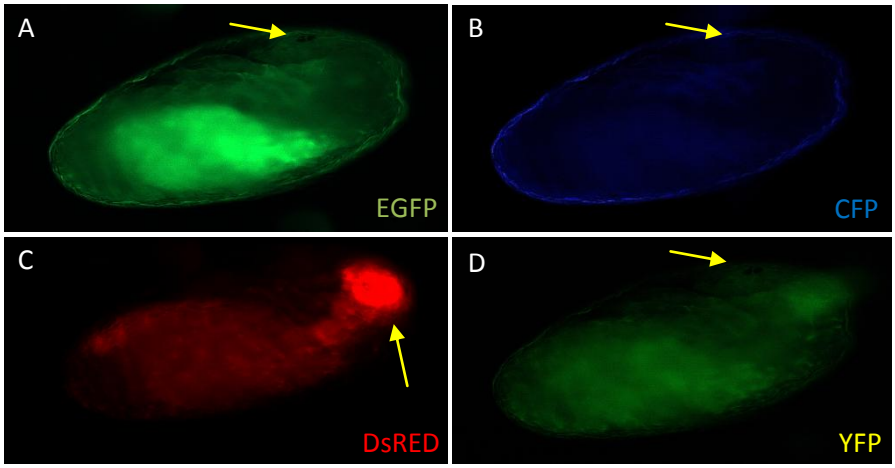

Figure 3.7.1 Lateral view of a transgenic untreated embryo from the cross CRE-16 x BB-215. Arrows show the expression of the transformation markers; (A, D) VW and (C) 3xp3 - DsRed. Embryo imaged on identical magnification on a fluorescent stereomicroscope with GFP, DsRed, YFP and CFP filters.

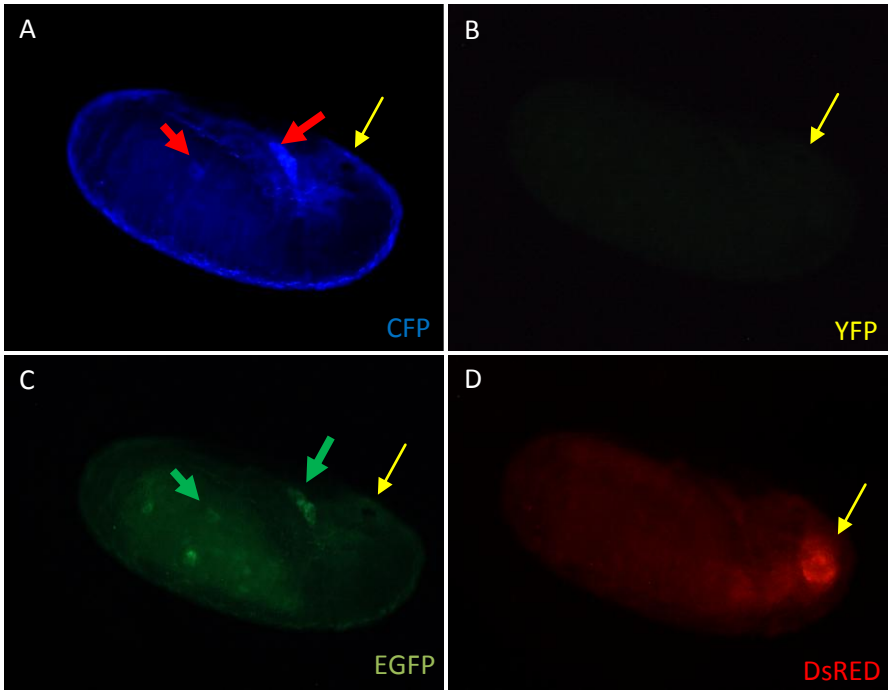

Figure 3.7.2 Lateral view of a transgenic embryo from the cross CRE-16 x BB-215, undergone heat shock treatment. Yellow arrows show the expression of the transformation markers; A, D) VW and C) 3xp3 - DsRed. Red arrows in image A show potential clones fluorescing in CFP UV filter. Green arrow shows emission of the CFP acquired by the GFP filter. Embryo imaged on identical magnification on a fluorescent stereomicroscope with GFP, DsRed, YFP and CFP filters.

3.5 Antibody staining with anti- EGFP and anti-DsRed antibodies

Antibody staining for the fluorescent proteins included in the *brainbow* construct took place, in order to observe clones at high magnification by confocal microscopy. Images shown below were taken from the progeny of the cross CRE-Voulis line mated with BB-215 line.

3.5.1 Transgenic embryos from cre or brainbow lines alone

In our endeavour to exclude, from our search for clones, unspecific staining, and to ensure that the antibodies worked on our fluorescent markers, we stained embryos from CRE-Voulis line and BB-215 line alone. In the figures that follow (*3.8.1 and 3.8.2*) an unspecific staining of anti-DsRed is depicted in the pleuropodia, on the first abdominal segment. No staining with the antibody for GFP/YFP/CFP was observed in the embryos of the *brainbow* line (*figure 3.8.1*), while the pleuropods were stained with anti-DsRed in both figures (*3.8.1 B and 3.8.2 A*).

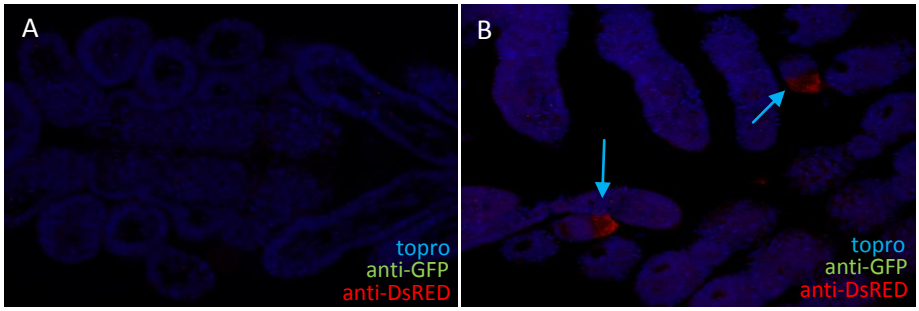

Figure 3.8.1 Ventral views of embryos from the *brainbow* line BB-215, focused (A) above and (B) below the appendages. Arrows in image B show the stained pleuropodia with anti-DsRed. Confocal microscopy. Antibody staining for GFP and DsRed. Topro was used to stain the nuclei.

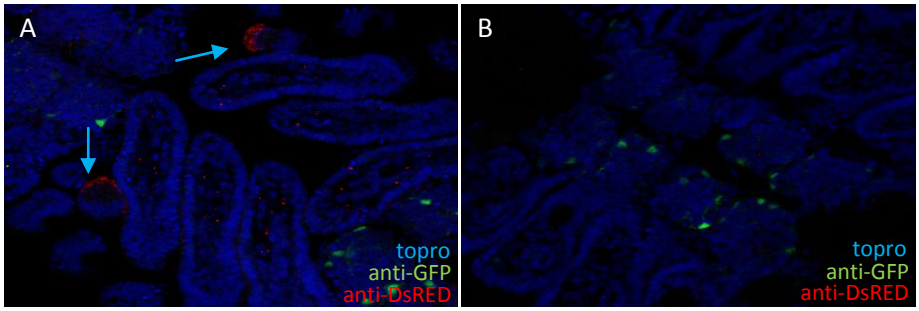

Figure 3.8.2 Ventral views of a transgenic embryo of the *cre* line CRE-Voulis, focused above and below the appendages. Arrows in image A show the stained pleuropodia with anti-DsRed.Confocal microscopy. Antibody staining for GFP and DsRed. Topro was used to stain the nuclei.

Additionally, I observed staining with the antibody for DsRed in the head lobes of late stage embryos carrying the 3xp3-DsRed marker, (*figure 3.8.3 A, B and C* and *figure 3.8.3 D, E and F*). This was due to the expression of the 3xp3-DsRed marker which has a different expression pattern from the 3xp3-EGFP one (*3.8.3 A and 3.8.3 B*). This expression pattern was observed in all transgenic lines.

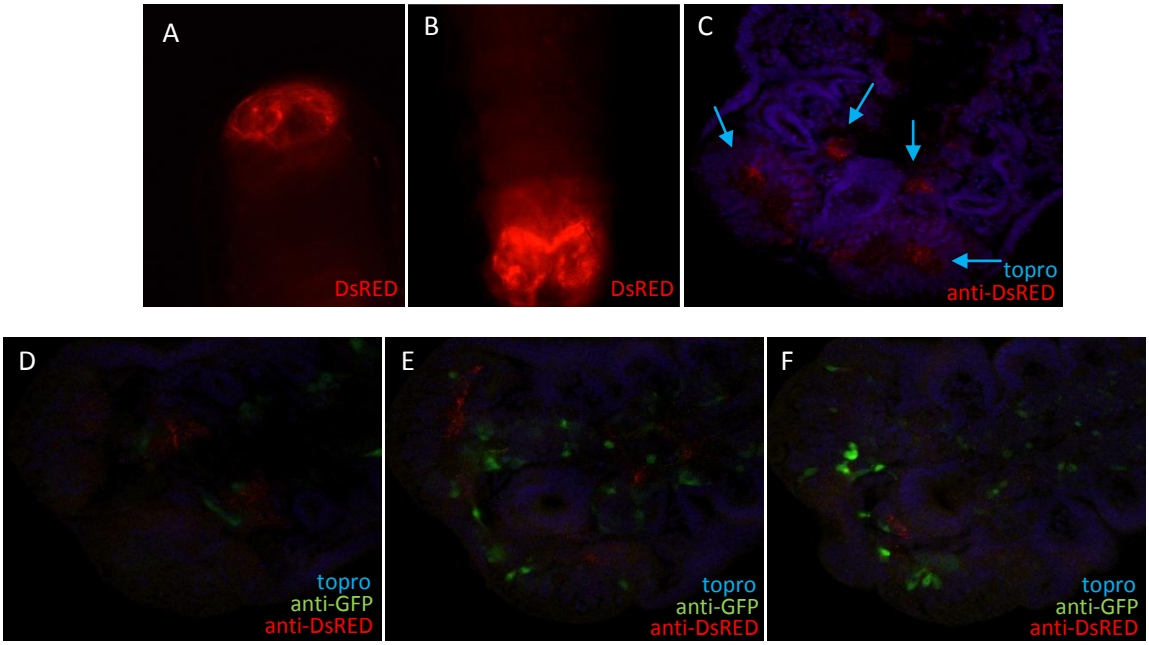

Figure 3.8.3 (A, B) Head lobes from a transgenic embryo and a transgenic larva of the *brainbow* line BB-215. Both imaged on a fluorescent stereomicroscope with DsRed filter. (C) Head lobe of a transgenic embryo stained with anti-DsRed and anti-GFP of the *brainbow* line BB-215; (D, E and F) Head lobe of a double transgenic embryo stained with anti-DsRed and anti-GFP of the CRE-Voulis x BB-215 line. Confocal microscopy. Topro was used to stain the nuclei.

3.5.2 Embryos carrying both cre and brainbow constructs, with and without heat shock treatment

Independently of whether the embryos were treated with heat shock or not, small asymmetrical clones were observed stained with anti-DsRed in all transgenic embryos carrying both *cre* and *brainbow* (*figures 3.9.2 and 3.9.3*). That came as no surprise, as our data previously showed that the heat shock promoter under which the expression of cre recombinase is mediated, is leaky. The clones were mostly no longer than 4 cells, apart from an embryo where 10 cells were observed (*figure 3.9.1*).

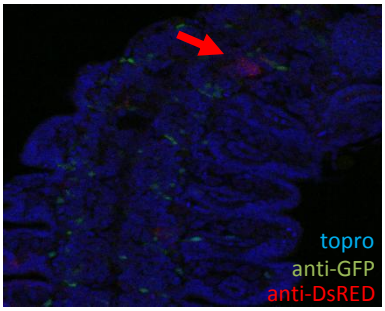

Figure 3.9.1 Ventral view of an embryo from the progeny of the cross CRE-Voulis x BB-215. (A) Image focused in the abdomen. Arrow in image shows a clone stained with anti-DsRed of 10 cells. Confocal microscopy. Topro was used to stain the nuclei.

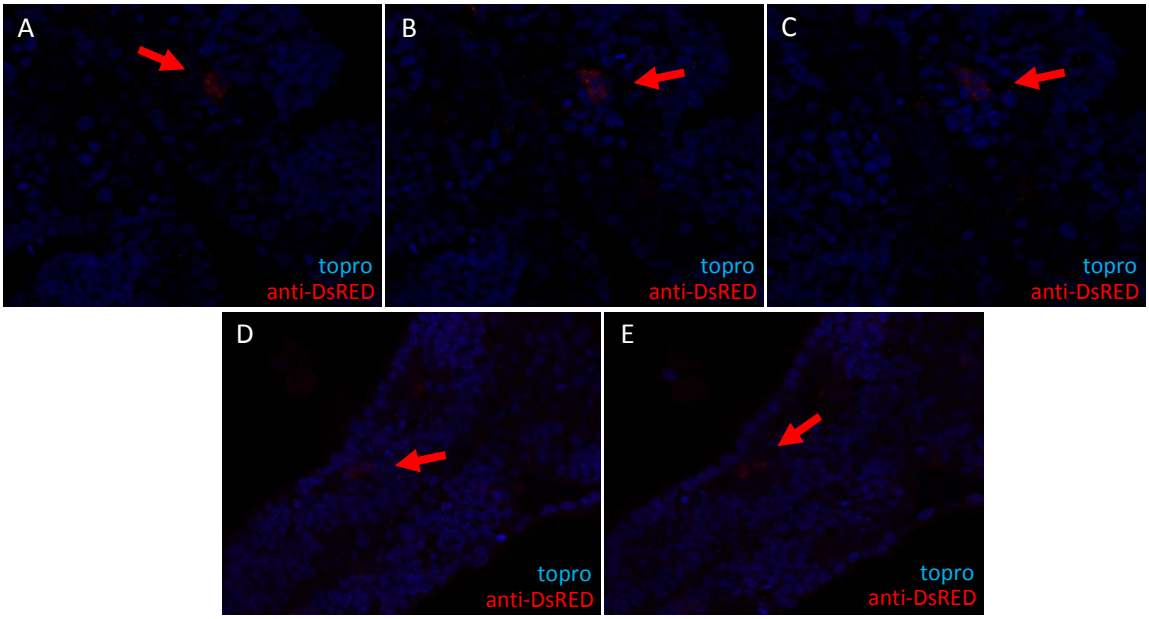

Figure 3.9.2 Ventral views of an embryo from the progeny of the cross CRE-Voulis x BB-215. (A, B and C) Images focused just below the thoracic appendages; (D and E) Images focused in the abdomen. Arrows show clones, of no more than 4 cells, stained with anti-DsRed. Confocal microscopy. Topro was used to stain the nuclei.

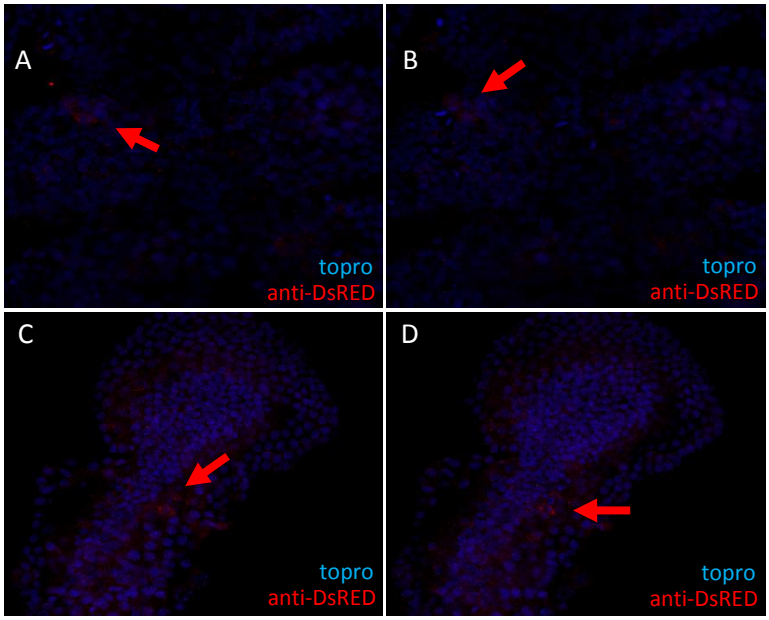

Figure 3.9.3 Ventral views of an embryo from the progeny of the cross CRE-Voulis x BB-215 (A, B) Images focused just below the thoracic appendages; (C and D) Images focused in the abdomen. Arrows show clones of no more than 4 cells, stained with anti-DsRed. Confocal microscopy. Topro was used to stain the nuclei.

4. Discussion and conclusions

In this project we have successfully implemented a protocol of *germline transformation* and henceforth in our lab transgenic lines in *Tribolium castaneum* can be reliably generated. I also made a first attempt to introduce *brainbow* to the emerging model arthropod *Tribolium castaneum* under the control of heat-inducible cre, as a system for generating marked genetic mosaics.

My results from the PCR experiments suggest that recombination is happening between all the variants of lox sites. In addition, confocal microscopy revealed cherry protein labelled clones, likely resulting from recombination between the *loxN* recombination sites. However, the observed asymmetrical clones are not only few and rare but also very small. No clone observed was ever more than 10 cells.

In conclusion, my results suggest that the cre/lox system is working in *Tribolium castaneum*. Recombination between the lox variants is happening. However, at present, either the recombination rate is too low, or there is a problem with fluorescent protein maturation, stability and/or localization. Finally, our results show that the heat shock promoter used to mediate *cre* recombinase expression is leaky, making it unsuitable for conditional induction of clones.

The system as it is cannot be used as a tool for the experiments it was designed for. It lacks the control of recombination events, and the formation of sufficient clones.

4.1 Possible explanations for the low efficiency of the genetic tool

There are numerous speculations one can make as to why the system is not as efficient as anticipated:

1. The apparent absence of fluorescent proteins could mean that all or some of the fluorescent proteins at my construct are not properly expressed. Insufficient expression could be caused by the localisation signals they bear. Maybe the proteins do not mature properly, are targeted for degradation and/or are mislocalised within the cell. The brainbow construct I used was derived from one designed for use in mice, with mammalian localization signals. When trying various genetic systems in different species, no matter how evolutionary close or far they are, we should always bear in mind that whatever worked for one might not immediately work for the other and that endogenous elements should, where possible, be favoured (Schinko *et al*, 2010).
2. The *cre* recombinase might be expressed at low levels or might take a long time to mature, hence the apparent low number of recombination events in my results.

3. Cells expressing any of the fluorescent proteins of the *brainbow* construct could be outcompeted by wild type cells, preventing them from forming bigger clones. There is also the possibility that the proteins expressed in recombined cells are toxic enough, to not only prevent them from proliferating, but also driving them to death/apoptosis.

4.1 Future prospects

In order to clarify where the problem lies, one could do in situ hybridization experiments for the mRNA molecules encoding the three fluorescent proteins. This will show if the genes encoding the fluorescent proteins are transcribed after recombination events within large and/or numerous clones of cells, suggesting the problem lies within translation and localisation. Alternatively, if the experiment above reveals that mRNA molecules encoding the fluorescent proteins are restricted to small and rare clones of cells, the explanation could lie in the low expression of cre. Low expression could be overcome by overexpressing cre recombinase by multiple insertions in the genome or with the use of a more powerful promoter.

One could also make an attempt to use another system other than cre/lox for generating clones, such as the flp/frt system, commonly used in *Drosophila melanogatser*.

Non fluorescent markers for transgenesis should also be included in any new constructs. Since *brainbow* uses 4 different fluorescent proteins, fluorescent markers can only make the search for clones harder to accomplish. It would also be wise to avoid, if possible, the use of the artificial 3xp3 promoter element which drives expression much more widely than the eye.

Finally, if everything in the system works sufficiently enough, but the promoter that drives the recombinase remains leaky, it could be changed. The heat shock promoter could be exchanged with one that does not require a change of temperature to express the gene but the presence or absence of a substance; a chemically regulated promoter. A system based on temperature can be too sensitive as changes in temperature are not always avoidable.

5. References

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