Towards a Novel Transgene Control Switch in Insects

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Declaration

I, Emily Frances M^{c}Gann, declare that except where indicated by specific reference, the work submitted is the result of my own investigation and that no portion of the work presented has been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

I would like to thank Karen,

for helping me with the angry fish and the spiky hedgehog

Abstract

Ligand-inducible transcriptional control devices that regulate ectopic gene expression both temporally and spatially are essential tools for the study of gene function. There are also real-world applications for such systems such as those employed by Oxitec Ltd. for insect pest control, where switch systems are used to control expression of effector proteins. This work presents the first steps towards developing two new small molecule responsive switch systems in insects.

The first is the auxin-inducible system taken from plant biology and adapted for use in *Drosophila melanogaster*. The target protein is tagged with an auxin inducible degron (AID), this is recognised by the plant protein TIR which is expressed ectopically. The interaction between the AID and TIR is mediated by the presence of the phytohormone auxin. When auxin is present TIR binds to AID and recruits the protein degradation machinery of the cell. Thus, silencing expression at the post-translational level.

The second utilises the synthetic devices created by Gitzinger *et al.* (2012) to design a similar system for use in the dengue fever vector *Aedes aegypti*. The designed system is responsive to vanillic acid and encompasses a trans-activator and an operator sequence. This represents a Van-Off system where vanillic acid is required to silence gene expression.

The components for both systems were successfully injected into their respective target organisms and transgenic lines established. Functional assays were conducted to determine the efficacy of the systems. The auxin responsive system as implemented here did not function as predicted however the vanillic acid system did turn on in *A. aegypti* pupae.

List of Abbreviations

AID	Auxin Inducible degron
ARE	Auxin responsive elements
ARF	Auxin response factors
bp	Base pair
CI	Cytoplasmic incompatibility
CRISPR	Clustered regularly interspaced short palindromic repeats
dNTP	Deoxynucleotide
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
Gal4BD	Gal4 protein binding domain
GSS	Genetic sexing systems
HDR	Homology directed repair
Heg	Homing endonuclease genes
IRS	Indoor residual spraying
LB	Lysogeny broth
LWT	Latin Wild Type
NHEJ	Non-homologous end joining
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
siRNA	Small interfering RNA
SIT	Sterile insect technique
TAE	Tris-acetate-EDTA
TALEN	Transcription activator-like effector nucleases
Tet	Tetracycline
TetO	Palindromic operator sequence recognised by TetR
TetR	Tetracycline responsive protein
Tris	tris(hydroxymethyl)aminomethane
tTA	Tetracycline responsive trans-activator
tTAV	Tetracycline responsive trans-activator

UAS	Upstream activating system
Van	Vanillic Acid
VanO	Palindromic operator sequence recognised by VanR
VanR	Vanillic acid responsive transcriptional repressor protein
ZFN	Zinc finger nuclease

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Chapter 1: An Overview of Ectopic Transgene Control in Insects

The temporal and spatial control of ectopic transgene expression is an essential component of modern genetic analysis. This is particularly apparent for studies involving *Drosophila melanogaster* where the development of modern genetic tools, as well as the abundance of sequence information has allowed for the analysis of a plethora of processes (Duffy 2002; Pfeiffer *et al.* 2010; McGuire *et al.* 2004).

These transgene "switches" can function at: the transcriptional stage by targeting DNA; the pre-translational stage targeting mRNA or at the post-translational stage by targeting the protein itself. There is an array of currently available systems that in some cases can be combined to form higher order networks to control multiple components at different times. This provides a more detailed picture of how a component functions within its system.

The ideal control system would act quickly, be highly target specific, have reversible effects, be fully repressed in the off state, and be 100% efficient and can be combined with many reporter and promoter sequences. Whilst no one system carries these traits there are many systems that demonstrate some of them. This chapter details the currently available systems for ectopic transgene control in insects looking predominantly at those available for *Drosophila melanogaster*. This provides a comprehensive backdrop for the work presented in this thesis.

1.1 GAL4/UAS and its Variants

The GAL4/UAS system is the most broadly used and characterized system for directed gene expression in *D. melanogaster*. It is a binary system with the ability to switch a gene on or off in a spatially specific pattern. It is primarily used to express a gene of interest in a spatially restricted manner. This technique is bi-partite comprising a separate driver and reporter element, no effect is seen until the two elements are both present in the same fly (e.g. after crossing two lines together). The reporter is transcriptionally silent until it is crossed to the driver. This allows for the study of dominant lethal phenotypes as the two components are maintained in separate strains. The gene of interest, the responder, is controlled by five tandemly arrayed Gal4 binding sites (the UAS). Transcription requires the presence of Gal4, without the ligand the target gene is maintained in a transcriptionally silent state. To activate transcription the responder line is crossed to a separate line that express Gal4 in a specific tissue or selection of cells, this is the driver line. The progeny of the cross will express the responder in

those tissues and cells where Gal4 is also expressed (Figure 1.1). As the responder line is transcriptionally silent until crossed to the driver this means that parental lines can encode gene products that are toxic or lethal. Predominantly this system provides tight spatial control over reporter gene expression as the GAL4 effector can be placed under control of any of hundreds of tissue specific promoters (Duffy 2002).

GAL4 was first identified as a gene regulator in *Saccharomyces cerevisiae* (Laughon *et al.*, 1984; Laughon and Gesteland, 1984). It controls transcription of galactose-responsive genes by binding to four specific 17 base pair (bp) sites upstream of the target gene. These four sites are referred to as the Upstream Activating Sequences (UAS) and are analogous to enhancer elements in higher eukaryotes (Duffy 2002). Fischer *et al.* (1988) demonstrated that the GAL4/UAS system could be used to spatially control target gene expression in *D. melanogaster.* The reporter in this example was a histochemical stain for β-galactosidase activity. When *lacz* is fused downstream of the *Adh* promoter it results in β-galactosidase production in four larval tissues: the fat body, malpighian tubules, anterior midgut and middle midgut. To demonstrate proof of principle that the GAL4 system could drive expression in *D. melanogaster* the *Adh* promoter *and lacz* were separated and combined with the components of the GAL4/UAS system. *lacz* is fused downstream of the UAS from *S. cerevisiae* and the core promoter fragment of Hsp70, the heat responsive elements have been removed. The driver comprised the *Adh* promoter upstream of Gal4. When the two lines were crossed GAL4 is produced in the four tissues associated with *Adh,* GAL4 binds to the UAS and drives expression of *lacz* which produces β-galactosidase. A histochemical stain for β-galactosidase in larval guts confirmed the expected spatial expression pattern and demonstrated the feasibility of this system for controlling spatial transgene expression in fruit flies (Fischer *et al.* 1988).

In 1993 Brand and Perrimon expanded the application for the Gal4/UAS system by studying developmental processes through the directed mis-expression of cloned genes. They demonstrated that the system could be used to study regulatory interactions during embryonic development by expanding the expression domain of *even-skipped*. The results of this demonstrated that it was a repressor of *wingless*. As mentioned previously the system is bi-partite. Brand and Perrimon (1993) constructed an enhancer trap Gal4 construct called pGAWB that led to the production of GAL4 driver lines that could target expression to nearly every major tissue type (Brand and Perrimon 1993).

Many of the driver lines available express throughout all developmental stages. If the gene of interest is lethal at early stages, then it because impossible to study its effects at later

developmental stages. Temperature can be used to bypass the deleterious effects at early stages. GAL4 activity is temperature dependent. Minimal activity is observed at 16^{o}C whilst 29^{o}C is considered to give the highest activity without compromised viability and fertility. Simply by controlling the rearing temperature a variety of expression levels of GAL4 can be achieved. Whilst providing good temporal control using temperature to activate the system has several drawbacks including: a basal expression level irrespective of temperature; transient transgene induction and the deleterious effects of prolonged heat shock (Halfon *et al.* 1997).

The repressor protein Gal80 can be used to control the temporal expression of Gal4/UAS. Gal80 binds to the Gal4 trans-activator domain preventing transcription of Gal4 inducible genes in *S. cerevisiae* (Elliott and Brand 2008). Gal80 can also repress Gal4 in *Drosophila* (Lee and Luo 1999). A commonly used variant is temperature sensitive Gal80 (Gal80ts), when under the ubiquitous promoter *tubulin 1α* Gal80ts represses Gal4 activity in all tissues. When the flies are reared at 30^{o}C for several hours the Gal80ts becomes inactive thus allowing Gal4 to bind the UAS and activate transcription (McGuire *et al.* 2003). This provides the researcher with good temporal control however the effect is still transient.

The Gal4-UAS system is used widely throughout the *Drosophila* research community, currently there are over 7800 Gal4 dependent lines available for order from the Bloomington Drosophila Stock Centre. The Kyoto Stock Centre has over 4000 Gal4 lines. Whilst Gal80 has its applications there are far fewer lines readily available for use; around 100 lines from Bloomington and 17 from the Kyoto centre.

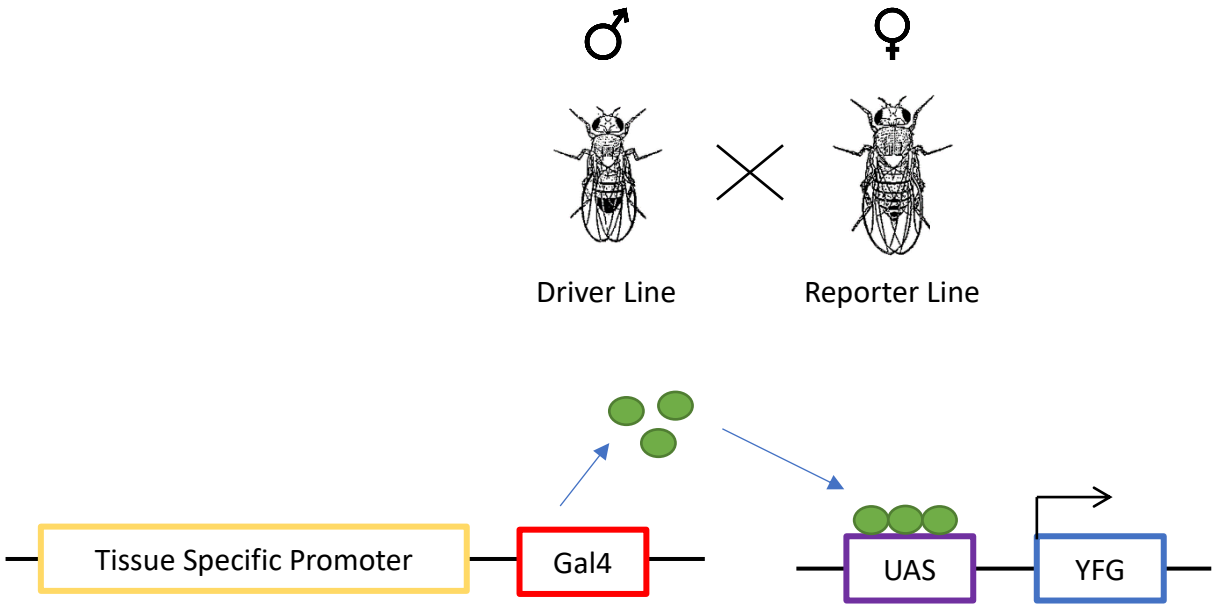


Figure 1.1
The Gal4 system is a bi-partite system used to express a gene of interest (Your Favourite Gene; YFG) in a specific sub-set of cells. The driver line carries the tissue specific promoter upstream of Gal4. The Reporter line carries the UAS upstream of YFG. In offspring carrying both constructs Gal4 is produced in a specific sub-set of cells. The Gal4 binds the UAS and drives expression of YFG.

1.2 LexA/Lexop

The repressor protein LexA from *Escherichia coli* in response to DNA damage LexA dimerises and binds to specific LexA DNA-binding motifs (LexAop) found upstream of target genes. Fusing the trans-activation domain VP16 from *Herpes simplex* to LexA (LexA TA) allows it to drive expression of genes that contain LexAop motifs within their promoters. LexA TA is placed under a promoter of choice. LexAop motifs are inserted into the promoter region of the target gene. Flies carrying both LexA and LexAop will express LexA in a specific subset of cells, it will bind to LexAop and drive expression of target genes (Pfeiffer *et al.* 2010). This system is orthologous to Gal4/UAS and both systems can be combined within the same fly however novel Gal4 reporter lines must be generated. This system is gaining some uptake in the community although it is not nearly as well used as the Gal4 system alone. Bloomington stock around 1600 lines that are compatible with LexA.

1.3 The Tetracycline Trans-activator (tTA) System

Small molecule responsive systems can be used to control transgene activation *in vivo*. The most well characterised system in *Drosophila* utilises the antibiotic tetracycline alongside the native response elements from *Escherichia coli*: the Tet repressor protein (TetR) and an operator sequence (TetO). In *Drosophila,* the TetR is fused C-terminally to the VP16 activation domain from *Herpes simplex* forming a tetracycline responsive trans-activator (tTA). In the absence of tetracycline, tTA binds to TetO and recruits the transcription machinery driving expression of downstream genes. When tetracycline is present it binds to tTA causing a conformational change in TetR. TetR can no longer bind to TetO and the gene is silenced (Urlinger *et al.* 2000) (Figure 1.2). This configuration is termed Tet-Off.

The system has been successfully modified to run in a Tet-On configuration (Figure 1.3). In Tet-On the TetR is mutated so that it will only bind to TetO in the presence of doxycycline, an analog of tetracycline. The reverse-TetR (rtTA) is activated by the addition of doxycycline it binds the TetO and activates transcription (Elliott and Brand 2008). Tet-On has a core advantage over Tet-Off in that it does not require the continuous feeding of an antibiotic to the animal. Its use in *Drosophila* was initially hindered by high background expression levels of the target gene, induction of target gene expression is also slow and inconsistent (Stebbins *et al.* 2001). These problems were addressed by the generation of rtTA-M2-alt (Urlinger *et al.*, 2000; Stebbins *et al.*, 2001).

In these two systems, the antibiotic is delivered in the food to the transgenic animal. This means that there is a latency period between exposure and an observed effect. Expression levels will also change as the ligand accumulates within the animal. Both the Tet-On and Tet-Off system have been demonstrated to be highly specific and offer tight control. As well as this they are dose dependent and highly responsive demonstrating significant suppression or induction when fed normal fly food containing 1 µg/ml of the appropriate antibiotic. Targeted gene expression using rtTA-M2-alt can be detected after 3 hours when larvae are fed food containing 1 mg/ml doxycycline. Induction of suppression times can be greatly reduced by injecting the ligand directly into the animal (Stebbins *et al.*, 2001).

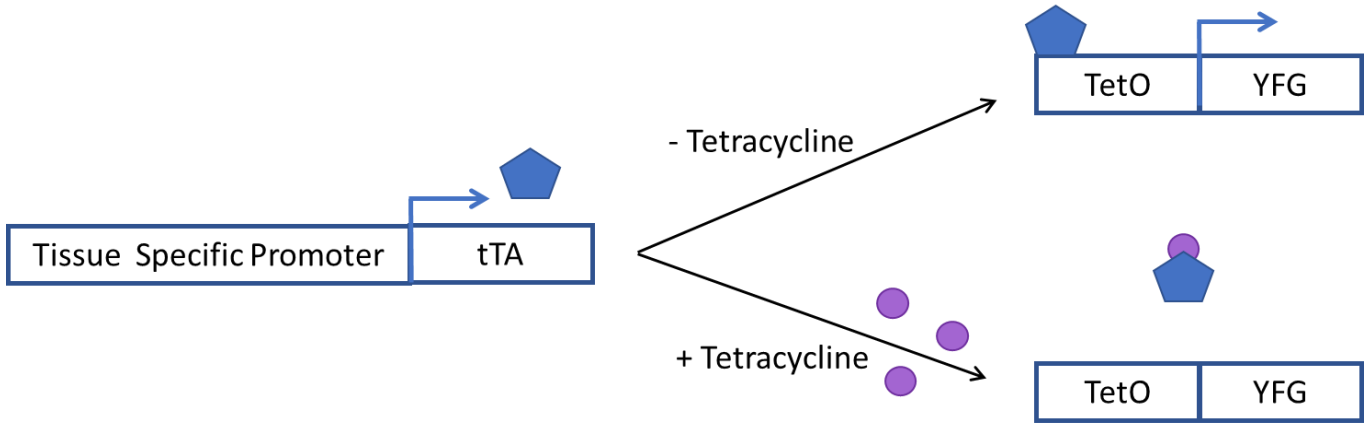


Figure 1.2
The Tet-Off system consists of the tTA (tetracycline trans-activator), driven by a tissue specific promoter, and its specific binding sequence (TetO). tTA is produced and binds to the TetO when there is no tetracycline in the organism. This drives expression of Your Favourite Gene (YFG). When the insects are fed tetracycline, it binds to the tTA and causes a conformational change that prevents it from binding to the TetO. This silences YFG.

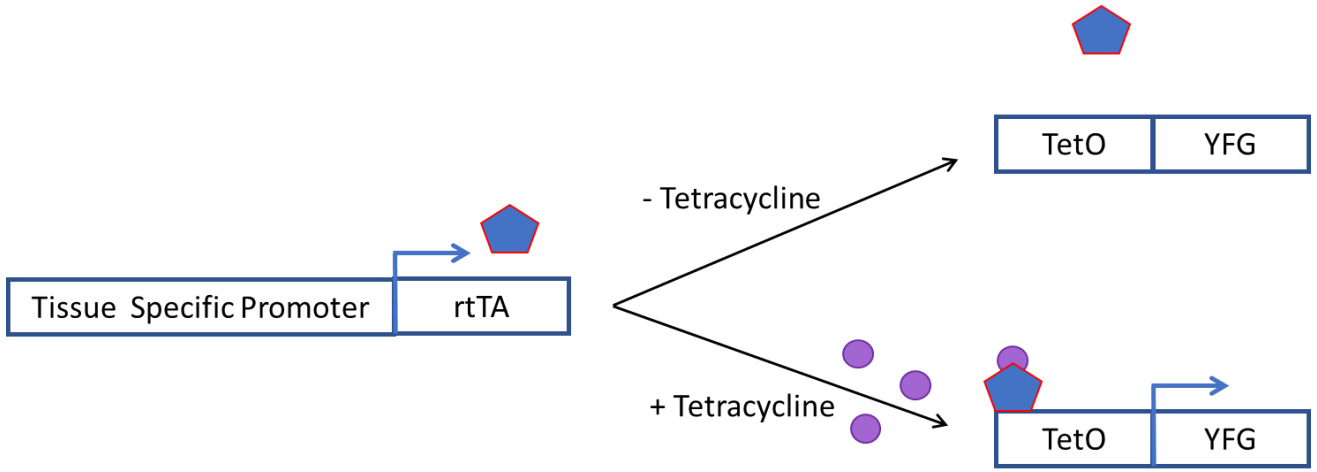


Figure 1.3
The Tet-On system depends on a mutated tTA termed rtTA. This mutation prevents rtTA from binding to the operator (TetO) unless there is tetracycline present. Your Favourite Gene (YFG) is only activated in the presence of tetracycline. YFG is silenced when insects are not fed tetracycline.

1.4 RNAi

RNA interference (RNAi) is a native cellular mechanism for silencing a gene *in vivo*, it forms a central part of the immune response to foreign genetic material. The RNAi mechanism is initiated by the presence of double-stranded RNA (dsRNA). This is cleaved by the ribonuclease Dicer. Dicer generates double stranded fragments 20-25 bp in length, these smaller fragments are termed small interfering RNAs (siRNAs). The siRNAs unwind into two single-stranded RNAs (ssRNAs), these are individually termed the guide strand and the passenger strand. The passenger strand is degraded, the guide strand binds to the RNA-induced silencing complex (RISC) and then binds to the target mRNA. The RISC complex blocks this sequence from being subsequently bound by the ribosome and cleaves it. This prevents translation and so knocks down gene expression (Schulz *et al.* 2009; Perrimon *et al.* 2010) (Figure 1.4).

RNAi has been used extensively to knock-down target gene expression in *Drosophila melanogaster* and other insect species. Synthetic dsRNA introduced into the organism can suppress the gene of interest. dsRNA, siRNA or hairpin RNAs can be supplied to initiate the knockdown of the target sequence. This technique has been applied to study a variety of processes in development, reproduction, behaviour and immunology.

In *Drosophila* dsRNA is most commonly delivered through transgenes or by injection into pre-blastoderm embryos. Here the gene encoding the dsRNA is under the control of a Gal4/UAS line to provide tissue specific expression. There are several problems associated with RNAi experiments mostly to do with variable efficacy of the technique between tissues, mode of delivery and the genes being targeted. The knock-down is also temporary and prone to off target effects yielding false results or results which can be difficult to interpret (Munkácsy *et al.* 2016).

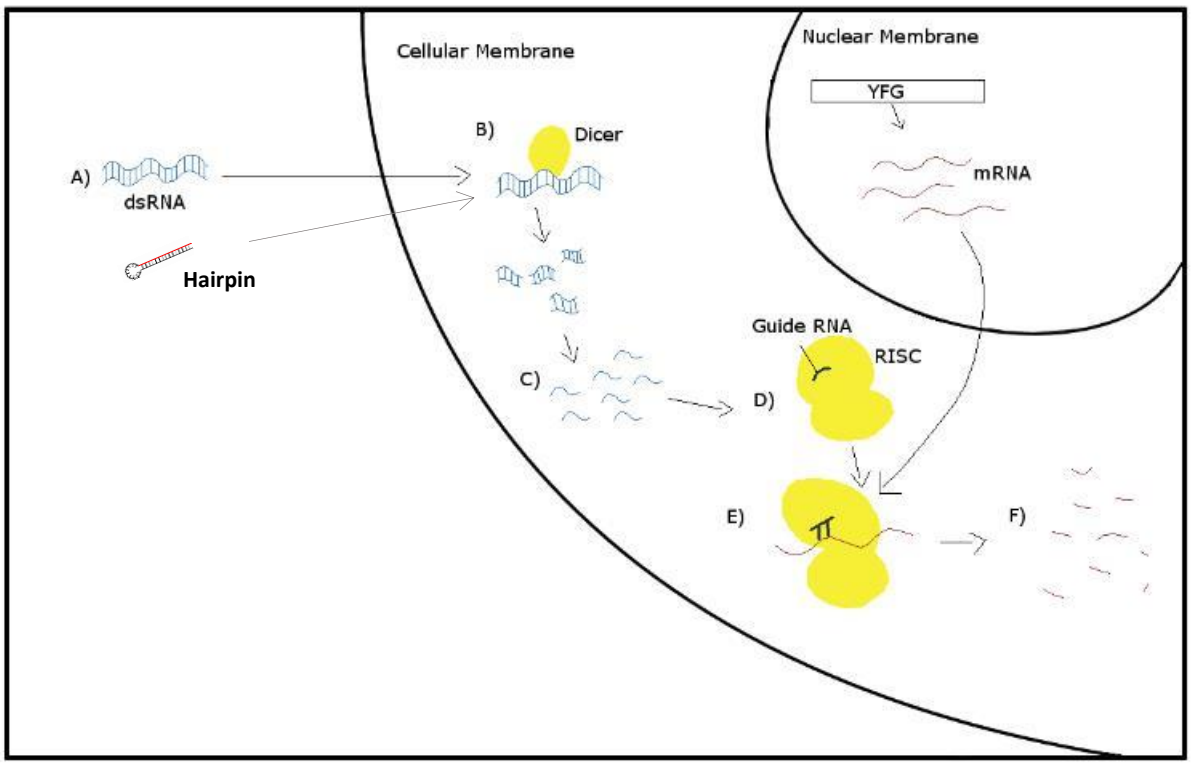


Figure 1.4

A schematic diagram of how RNAi functions.

A) Double stranded RNA (dsRNA) or Hairpin RNA is introduced and is moved into the cell through the cellular membrane.

B) The RNase DICER binds to the dsRNA or Hairpin RNA and cleaves it into fragments 20-25 bp long generating short interfering RNAs (siRNAs)

C) The siRNAs split into the guide strand and the passenger strand. The passenger strand is degraded.

D) The guide RNA is incorporated into the protein complex RISC.

E) mRNA from the gene of interest (YFG) is incorporated into RISC as the guide RNA serves as a template for the RISC complex

F) The mRNA is cleaved at these sites functionally silencing YFG.

1.5 Genome Editing

It is possible to modify the target gene directly using genome editing techniques. Sequence directed nucleases generate double stranded breaks in the DNA which are repaired either by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is a ligation process that is quite error prone resulting in small insertions or deletions (indels) at the target cleavage site, by targeting gene open reading frames this can lead to frameshift mutations. HDR by comparison requires a repair template, this homologous piece of DNA is used to generate a precise repair. The target supplied can be used to insert nucleotide specific alterations in the gene of interest, or even insert a large fragment into a specific locus flanked by two homology arms (Ren *et al.* 2017).

Earlier genome editing methodologies used pairs of either zinc-finger nucleases (ZFNs) (Bibikova *et al.* 2002; Rong and Golic 2000) or transcription activator-like effector nucleases (TALENs) to generate double stranded breaks in target sequences (Liu *et al.* 2012). These have drawbacks though as the ZFN and TALEN proteins must be individually engineered for the desired genome modification, for TALENs the entire process from TALEN construction to screening of mutants takes about a month (Liu *et al.* 2016). This has hindered the up-take of the technology in the field however both methods have been effectively applied to *Drosophila* research.

More recently the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR activating sequences) 9 system which forms part of the immune response in bacteria has gained favour as the leading method for genome editing. The system requires the nuclease Cas9 and a chimeric guide RNA around 100 nucleotides in length (sgRNA). The guide RNA is a fusion of two individual RNAs, crRNA and tracrRNA. crRNA binds to a 20-nucleotide region within the DNA. tracrRNA complexes with the crRNA and aids its incorporation into the Cas9 complex. sgRNA binds to the DNA next to a PAM (protospacer adjacent motif) of NGG, Cas9 is recruited and cleaves both DNA strands within the target sequence. The resulting double strand break is then repaired by the cell using either HDR or NHEJ (Bassett and Liu 2014).

The first successful CRISPR mutagenesis in *Drosophila* was reported in 2013 and were published by a several research groups at around the same time (Bassett *et al.* 2013; Gratz *et al.* 2013; Kondo and Ueda 2013; Ren *et al.* 2013; Sebo *et al.* 2014; Yu *et al.* 2013). Gratz *et al.* (2013) successfully altered the *yellow* gene and this mutation was transmitted to subsequent generations. Here two plasmids were co-injected into pre-blastoderm embryos one expressing Cas9 under the *Hsp70* promoter and the other expressing the sgRNA driven by the RNA polymerase III-dependent promoter from *U6*. The gene knock out efficiency was low with only

5.9% of injected embryos giving rise to mutant offspring. Higher mutagenesis efficiencies were achieved by co-injecting Cas9 mRNA and sgRNA into early stage embryos. The Cas9 is transcribed *in vitro* and due to the inefficient nature of NHEJ a much higher rate of mutagenesis is achieved, around 80% (Bassett *et al.* 2013, Yu *et al.* 2013). Alternatively, independent transgenic lines can be established where Cas9 is expressed in the germline under the *nanos* promoter and the sgRNA is ubiquitously expressed under the *U6* promoter (Kondo and Ueda 2013). When crossed around 90% of flies will give rise to mutant offspring. This process is efficient but time consuming as an individual transgenic line is required for each sgRNA, the Cas9 and sgRNA transgenes must also be removed after generation of the mutation. Finally, the sgRNA-containing plasmids can be injected into embryos where the Cas9 gene is expressed specifically in the germline either under the *nanos* or *vasa* promoters. Here only one transgenic line needs to be maintained. Whilst this method generated a high level of mutagenesis many of the injected flies were infertile (Sebo *et al.* 2013, Ren *et al.* 2013).

Whilst CRISPR is a faster method for genome editing there is a high chance of off-target effects. This is due to the relatively low targeting specificity of the sgRNA (Wei *et al.* 2013). The sgRNA will bind despite several mismatches within the 20-nucleotide target sequence and still cleave the DNA (Fu *et al.* 2013; Mali *et al.* 2013). This can be mitigated by careful site selection. The specificity can be increased by using a mutated Cas9 protein that can only make single strand breaks in the DNA. This “double-nick” approach requires two specific sgRNAs, each delivering a Cas9 protein to the target site, to generate a double strand break (Mali *et al.* 2013; Ran *et al.* 2013).

1.6 Generating Higher Order Networks

The systems described above provide a toolkit that can be applied to a variety of experimental questions. Whilst each tool on its own has a certain power and application many of them have been combined to give additional control to the system being explored. This could be through improving location specificity, providing tighter temporal control or adding a switch that can effectively turn other functional components (proteins) on or off. This may be through degradation of mRNA or the protein itself or causing conformational changes that render the protein inactive.

As the most commonly used tool for spatial control, the Gal4/UAS system has been partnered with many other systems, notably the tetracycline system and other small molecule responsive systems. There are two Gal4-hormone chimeras currently used in *Drosophila*, Gal4-estrogen (Han *et al.* 2000) and GeneSwitch (Roman and Davis 2002). These two systems depend upon feeding estrogen or progesterone respectively to the flies to induce binding of the chimeric Gal4 protein to the UAS, initiating transcription (Duffy 2002). Whilst this provides tight temporal control and the effects are reversible, the ligand can simply be removed from the diet, it requires the generation of additional driver lines contain the receptor for the Gal4-hormone chimera (Elliott and Brand 2008). Tet-On and Tet-Off can be successfully linked to Gal4/UAS. The target gene is placed under the TetO, and rt-TA-M2-alt under the UAS. The desired Gal4 driver can then be crossed in. Gal4 drives expression of rt-TA-M2-alt in a specific subset of cells. In Tet-On this can only bind to the TetO and activate transcription in the presence of tetracycline. In Tet-Off the rt-TA-M2-alt will only activate transcription when tetracycline is absent (Duffy 2002; Elliott and Brand 2008; Stebbins *et al.* 2001). This provides tight temporal and spatial control. The tTA and Gal4/UAS systems provide a remarkable level of control over transgene expression however it would be beneficial to have an orthogonal system that functions alongside existing driver lines. This would provide another tier of control to pull apart and manipulate gene function within complex developmental problems. The Gal4/UAS system has also been combined with LexA trans-activators, this combination works similarly to the Tet-On/Tet-Off system (Yagi *et al.* 2010), although as it is not ligand-responsive it is not reversible.

1.7 Novel Small Molecule Switches in Insects

This work details progress made towards two small molecule control switches. One was designed for initial use in *Drosophila melanogaster*, the other was tested in the dengue fever mosquito *Aedes aegypti*. These systems were developed to complement existing transgene control systems, aswitch allowing for tight temporal control of transgene expression. When selecting which small molecule systems to develop during this project it was important to understand the desires of the end user. The key desirable traits of a transgene control switch were summarised well by McGuire *et al.* (2004). They state that systems which control transgene expression should have the following four characteristics:

1) An inert inducer that does not harm the insect and is completely specific to the system itself
2) Control over the expression level by varying the ligand concentration
3) Rapid kinetics with immediate transcription following exposure to the ligand and turn off being limited by the decay rates of any pre-existing mRNA and the proteins themselves
4) A complete off-state with no basal expression in the absence of the inducer

The first chapter describes an auxin-responsive switch in *Drosophila melanogaster*. Initially this system was combined with the Gal4/UAS system. After this work took place a successful auxin-responsive system, conceptually very similar to the one trialled here, was published by Trost *et al.* (2016).

The second switch utilises vanillic acid in a system orthogonal to the Tet-responsive system. This system was designed to complement the suite of technologies currently employed by Oxitec, a world leader in genetic based pest management control systems.

Chapter 2: An Auxin Inducible System in *Drosophila melanogaster*

2.1 Introduction

2.1.1 The Auxin Signalling Pathway

Auxins are a small class of phytohormones that regulate plant growth and development by inducing targeted gene expression (Mockaitis and Estelle 2008; Gray *et al.* 2001; Abel and Theologis 1996). Auxins trigger expression of a diverse array of genes at low cellular concentrations within minutes of exposure (Tian *et al.* 2002; Goda *et al.* 2004). Gene expression is controlled through the post-translational degradation of a group of transcriptional repressor proteins called the Aux/IAAs (Figure 2.1). These proteins bind to auxin response factors (ARFs). In the absence of auxin the Aux/IAA binds to the ARF inhibiting transcription (Mockaitis and Estelle 2008). When auxins are present, they bind to the Aux/IAA and recruit the native degradation proteins of the cell. This relieves repression of the ARFs, they are free to bind to auxin responsive elements and induce transcription of auxin responsive genes. The auxins recruit the SCF (Skp-1-Cul1-F-Box) protein complex at the F-Box domain protein TIR1. The binding of auxin stabilises the interaction between TIR1 and the auxin inducible degron (AID) located within the Aux/IAA repressor protein (Hayashi 2012). The SCF^{TIR1} complex contains an E2 ubiquitin ligase, once the substrate has been stabilised this enzyme can polyubiquitinate the AID sequence. This serves as a signal for the Aux/IAA to be degraded by the 26S proteasome (Nishimura *et al.* 2009). Following degradation of the Aux/IAAs transcription of auxin dependent genes can occur (Figure 2.2).

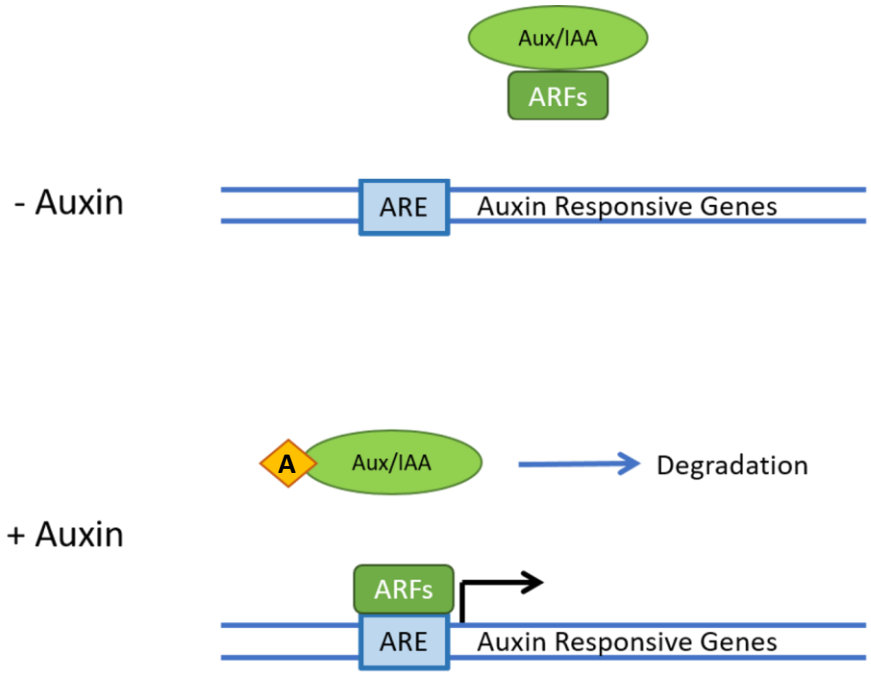


Figure 2.1

-Auxin: The Aux/IAA proteins bind to ARFs (Auxin responsive factors) and prevent them from initiating transcription of genes downstream of auxin responsive elements (AREs).

+Auxin: When the small phytohormone auxin is present in the cell (yellow diamond [A]) it binds to the Aux/IAA and targets it for degradation by the cell. This relieves the repression of ARFs and they can recruit transcriptional machinery to the ARE and activate transcription of auxin responsive genes.

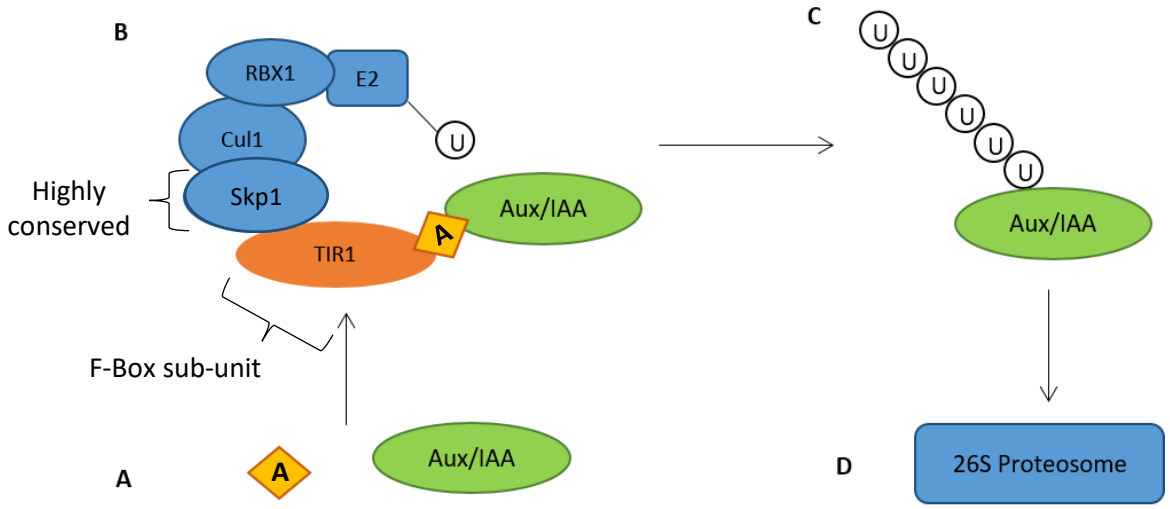


Figure 2.2

A: Auxin (yellow diamond [A]) binds to Aux/IAA repressor proteins

B: This stabilises the interaction between the Aux/IAA protein and the F-Box protein TIR1. In the absence of auxin these two proteins have very low affinity for one another. TIR1 recruits the cellular SCF complex at the c-terminal end of Skp1. This domain is highly conserved among eukaryotes. At which point the ubiquitin ligase domain of TIR begins to ubiquitinate the Aux/IAA repressor

C: The Aux/IAA is released with a poly-ubiquitin tail which signals it for degradation

D: The Aux/IAA is degraded by the proteasome allowing for transcription of auxin responsive genes

2.1.2 Auxin Inducible Protein Degradation

This pathway lends itself to exploitation in non-plant species. All eukaryotes have multiple forms of the SCF complex, the subunits of which are highly conserved between species (Nishimura *et al.* 2009). The variable F-Box subunit TIR1 confers substrate specificity and serves to anchor the substrate close the ubiquitin ligase (Ho *et al.* 2006). The TIR1-AID (auxin inducible degron) interaction is only found within plant species (Holland *et al.* 2012) however ectopically expressed TIR should still recruit the native Skp1 machinery. SCF^{TIR} promotes the degradation of AID tagged proteins in the presence of auxin. If a system expressed TIR and a protein tagged with the AID, the tagged protein should be rapidly depleted *in vivo* after the addition of auxin to the system (Nishimura *et al.* 2009).

This system would function similarly to that described above and is a copy of the biological system seen in plant species. The AID sequence is added to the end of the target protein sequence. When this sequence is translated, the protein will carry the AID tag. If the individual also expressed TIR then in the presence of auxin it would bind with high affinity to the AID. The AID tag would be polyubiquitinated and the target protein would be targeted for degradation by the proteasome. This would silence the protein. In the absence of auxin then AID and TIR have a low affinity and the protein would be expressed (Figure 2.3).

This system provides many advantages for rapid depletion of proteins. It is a direct approach whereas many techniques deplete proteins indirectly at the mRNA or DNA level. The difficulty with pre=translational centred techniques is that they are irreversible and can suffer from leakiness and off-target effects (Nishimura *et al.* 2009; Holland *et al.* 2012). The AID-approach is reversible allowing for the effects of depletion to be studied across a short time-frame. As AID is not endogenously expressed in non-plant species the system is highly specific. The AID system has been successfully deployed in yeast as well as several mammalian cell lines including chicken, hamster, mouse and human cells (Nishimura *et al.* 2009). Auxins induced rapid depletion of the target protein and this effect was reversible and specific (Holland *et al.* 2012). The system has been optimised in yeast with the truncation of the AID sequence and the creation of several vectors that incorporate key fluorescent markers and antibody tags (Morawska and Ulrich 2013). At the time this initial work was carried out an auxin switching system had not been adapted for use in whole animal studies.

This system would be an ideal addition to the current suite of molecular techniques available for use in *Drosophila*. It would allow for protein depletion to be studied at all life stages and the effect would be silent until the auxin supplement was introduced. Work by Turkel (Unpublished) demonstrated that the synthetic auxin 2,4-D was non-toxic to adult flies even at high concentrations (1000 μM) and was suggested as the most suitable auxin for future auxin inducible systems in *Drosophila*.

2.1.3 Auxin Inducible Protein Dimerization

An alternative application of the auxin system is to use it to control protein dimerization *in vivo*. In this case one of the proteins being studied would be tagged with the AID sequence. The other protein would be co-translated with the inclusion of modified TIR by a T2A linker bridge to allow the proteins to fold naturally. The modified TIR lacks the ubiquitin ligase domain and thus cannot polyubiquitinate the AID sequence. In the presence of auxin, the TIR protein and the modified TIR would be brought together causing the target proteins to dimerise. They would not be degraded as the TIR cannot add a ubiquitin tail to the AID tag. In the absence of auxin the TIR binding domain and the AID sequence have a low affinity for one another and so there shouldn't be a significant amount of dimerization (Figure 2.4).

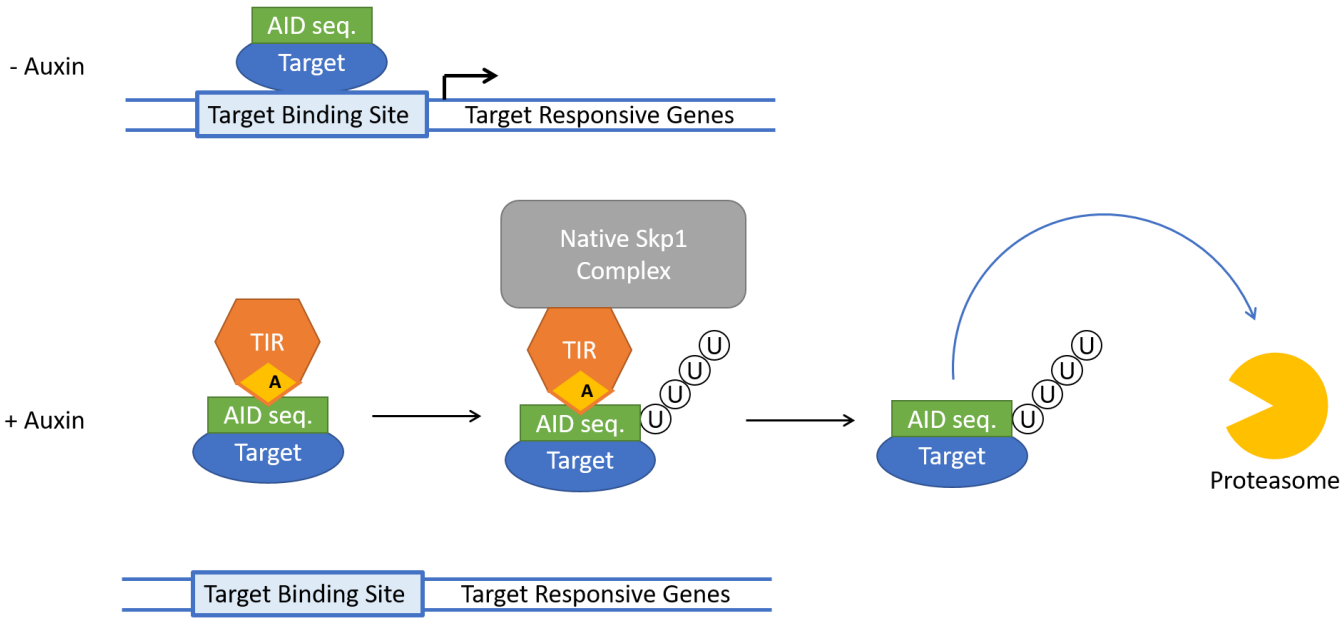


Figure 2.3

The AID sequence located within the Aux/IAA protein is sufficient to bind to TIR. In a system where a target protein carries the AID sequence and TIR is expressed then the degradation of the target should be responsive to auxin.

-Auxin: In the absence of auxin the target binds to its binding site and activates transcription of the target responsive genes. TIR is also produced however in the absence of auxin they have a low affinity for one another.

+Auxin: When auxin (yellow diamond [A]) is present it stabilises the interaction between the AID sequence and TIR. TIR then recruits the native Skp1 complex of the cell and the AID sequence is poly-ubiquitinated. The AID-Target protein is released from the TIR-Skp1 complex and is degraded by the proteasome.

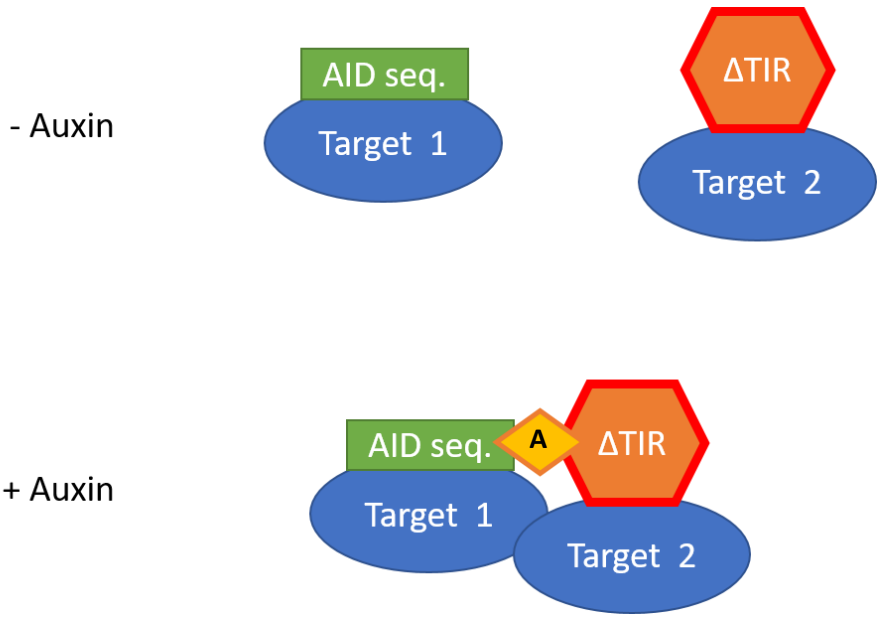


Figure 2.4

An auxin responsive system could be used to control protein dimerization *in vivo*. One target contains the AID sequence and the other target is fused to a modified TIR (ΔTIR) which lacks the ubiquitin ligase. This will prevent the AID tagged target from being degraded.

-Auxin: In the absence of auxin the AID sequence and ΔTIR have a low affinity for one another

+Auxin: When auxins are introduced to the system the interaction between the AID sequence and ΔTIR is stabilised. This brings the two targets close enough together to dimerise.

2.1.4 Initial Constructs Provided by Oxitec

Prior to this work Oxitec, a company specialising in genetic control systems for insect pest species, provided constructs for three auxin responsive systems to be tested in *Drosophila melanogaster*. In these constructs the AID sequence was taken from *Arabidopsis thaliana* and the TIR sequence from *Oryza sativa*. All three constructs are marked with a 3xP3 promoter upstream of DsRed2. 3xP3 is an artificial promoter comprised of three binding sites for Pax6 followed by a TATA box (Berghammer *et al.* 1999). In *Drosophila* 3xP3 is expressed specifically in the eyes and across all developmental stages. The combination of 3xP3 with the fluorescent protein DsRed2 allows for easy detection of transgenics. Construct maps are provided in Appendix 1.

2.1.4.1 Auxin Inducible Degradation of Gal80

#4759 (TIR/Gal80-AID) (Figure 2.5) encodes an auxin responsive system for the degradation of the Gal80, an inhibitor of the Gal4/UAS system. This single construct contains the TIR with an α-tubulin promoter which will ensure transcription occurs ubiquitously. The TIR sequence is followed by a nuclear localisation sequence and a c-myc epitope tag. There is then a ubiquitin bridge followed immediately by the AID-Gal80 sequence. The entire sequence is transcribed however during translation the ubiquitin is cleaved and the TIR protein is released, then the AID-Gal80 sequence is translated generating two distinct protein products from a single sequence.

When flies carrying the TIR/Gal80-AID construct are crossed to flies carrying a Gal4/UAS system and are reared without auxin, then stable Gal80-AID should be produced. This would inhibit Gal4 and so there should be no activation of genes downstream of UAS sequences. The Gal4 system is thus silenced. When progeny carrying both systems are reared with auxin present in the food TIR and Gal80-AID should bind with high affinity. The AID sequence would be poly-ubiquitinated and Gal80 would be degraded by the proteasome. Gal4 would then be free to bind the UAS and drive expression of target genes (Figure 2.6)

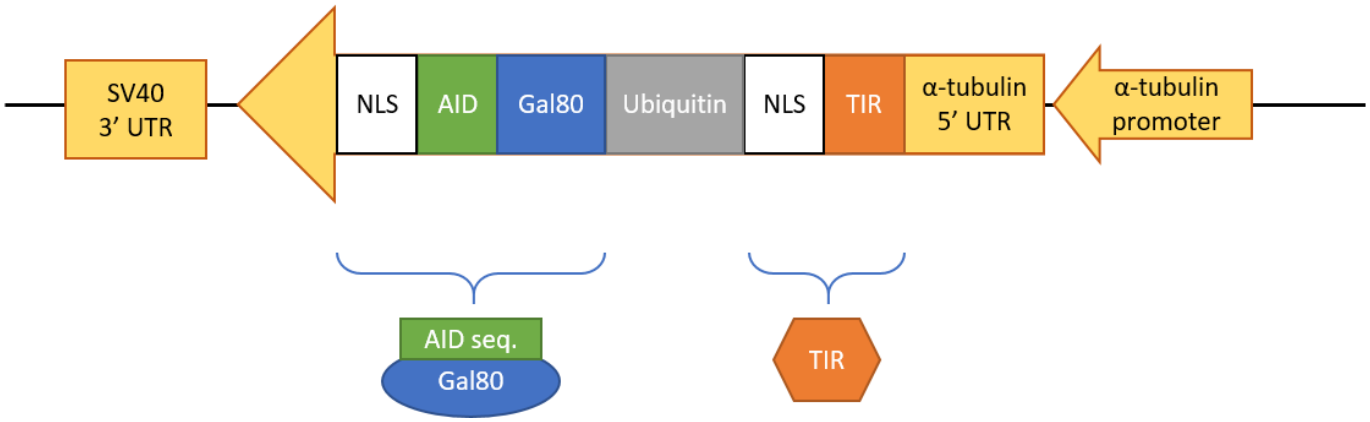


Figure 2.5

A schematic diagram of construct #4759 (TIR/Gal80-AID). The construct is driven by an α-tubulin promoter. The construct is transcribed into a single mRNA. During translation the ubiquitin is cleaved releasing the first protein however the translational machinery does not disassociate from the transcript and so the second fusion protein is translated. The first protein released is TIR. The second protein is Gal80 with an AID tag at the 3' end, both proteins also carry a nuclear localisation sequence (NLS).

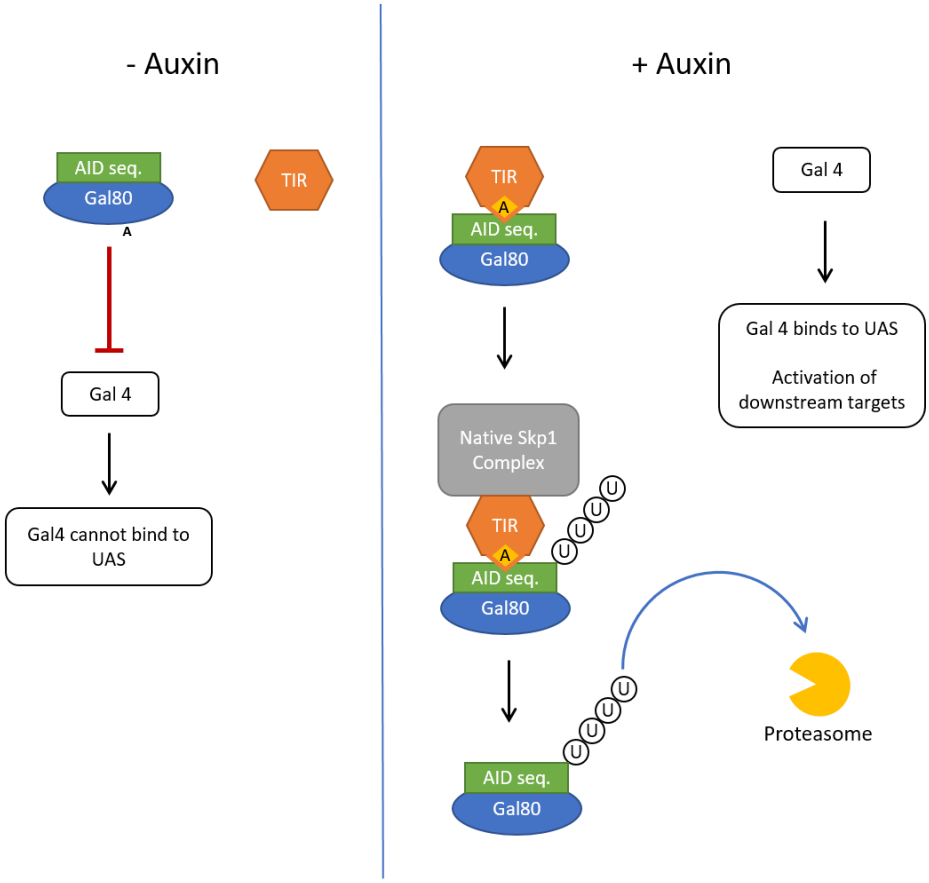


Figure 2.6

#4759 (TIR/Gal80-AID) produces AID tagged Gal80 an inhibitor of the Gal4/UAS system. Flies carrying both the TIR/Gal80/AID system and a Gal4/UAS system should be responsive to auxin in the food.

-Auxin: Gal80-AID and TIR have low affinity for one another. Gal80 inhibits the binding of Gal4 to the UAS and genes downstream of the UAS are silent.

+Auxin: Auxin (yellow diamond [A]) stabilises binding between TIR and the AID sequence. The native Skp1 complex of the cell is recruited and the AID sequence is poly-ubiquitinated. This targets Gal80-AID for degradation by the proteasome. As Gal80 is degraded Gal4 can bind to the UAS activating transcription of downstream target genes.

2.1.4.2 Auxin Inducible Dimerization of Gal4

#4775 (Gal4BD- ΔTIR /VPI6-AID) (Figure 2.7) is a single construct that encodes a system for auxin inducible protein dimerization. The Gal4 protein binding domain (Gal4BD) is separated from the trans-activator domain (VP16). The VP16 is tagged by AID. The Gal4BD is linked to a modified TIR protein (ΔTIR). The modified TIR sequence lacks the ubiquitin ligase domain. Once again there is a ubiquitin bridge between the Gal4BD- ΔTIR and VP16- AID. This bridge is cleaved co-translationally and results in two distinct protein products.

In the presence of auxin, the AID and ΔTIR should have a high affinity for one another. This would cause the dimerization of the Gal4BD and VP16. The dimerised protein would recognise the UAS and recruit the transcriptional machinery of the cell. This would lead to the expression of transgenes downstream of the UAS. The degradation machinery is not recruited in this case as the ubiquitin ligase domain is absent. In the absence of auxin Gal4BD- ΔTIR and VP16-AID should have low affinity. The Gal4BD still binds to the UAS however the trans-activator domain is missing and so there should be no transcription of target genes (Figure 2.8).

2.1.4.3 Auxin Controlled Male Sterility

#4789 (TIR/mCherry-Protamine-Fok1-AID) (Figure 2.9) encodes a system that in the absence of auxin should generate infertile males. Again, this system is encoded on a single construct the promoter is β2-tubulin which limits expression to the testis. There is an ubiquitin bridge between TIR and mCherry-Protamine-Fok1-AID. This is cleaved away during translation. The second protein product is a large fusion protein. mCherry serves as a marker protein for the fusion protein. The protamine portion is involved in chromatin condensation in late stage spermatids ensuring localisation to the nucleus at the correct time. Fok1 is a non-specific DNA nuclease enzyme that forms double stranded breaks in the DNA. The fusion protein is tagged with the AID sequence that can be recognised by TIR.

In the absence of auxin mCherry-Protamine-Fok1-AID would be free to cleave the DNA of late stage spermatids. This should generate non-viable sperm and so these males are predicted to be sterile. In the presence of auxin TIR and mCherry-Protamine-Fok1-AID would be brought together. The AID sequence could then be poly-ubiquitinated and the entire fusion protein degraded by the proteasome. In this instance the DNA would not be cleaved and the male would have viable sperm (Figure 2.10)

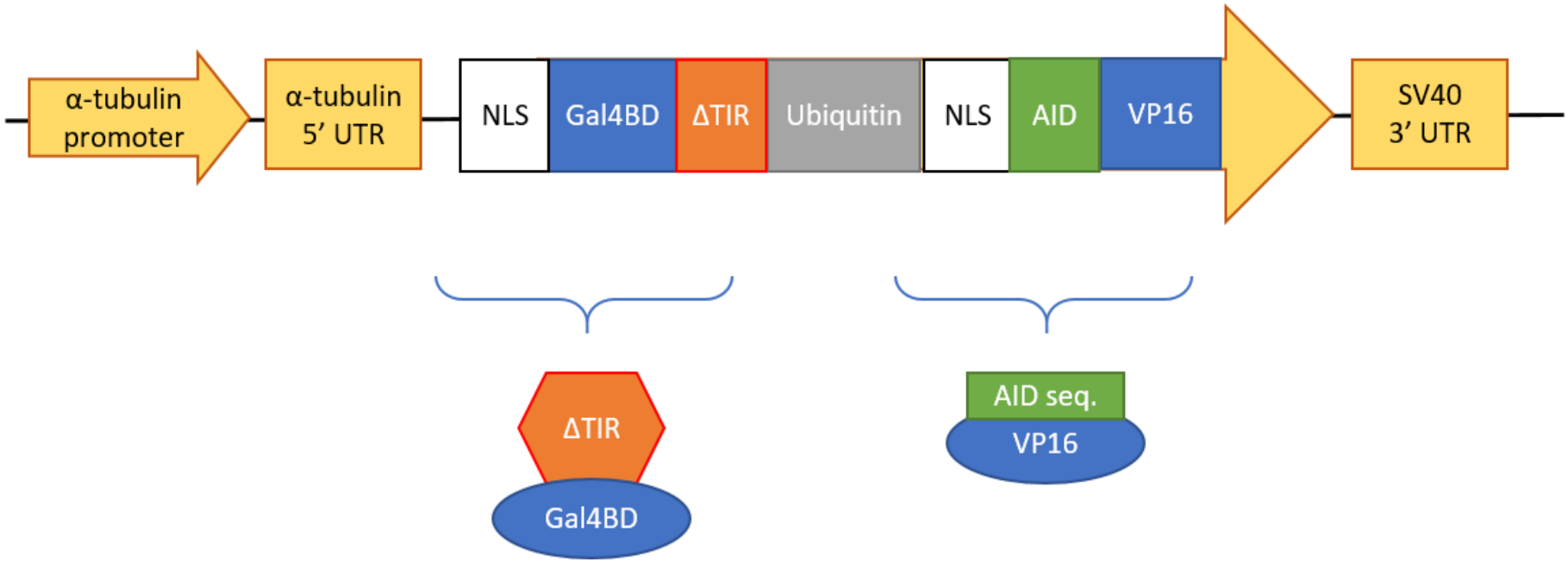


Figure 2.7

Schematic diagram of #4775 (Gal4BD-ΔTIR/VPI6-AID). This construct is driven by the α-tubulin promoter. The construct encodes a single transcript that is translated into two separate proteins. The first fusion protein is Gal4BD-ΔTIR, this is the binding domain for Gal4. The second is VP16-AID, this is the trans-activator domain tagged with AID. There is a nuclear localisation signal (NLS) located at the 5' end of each fusion protein.

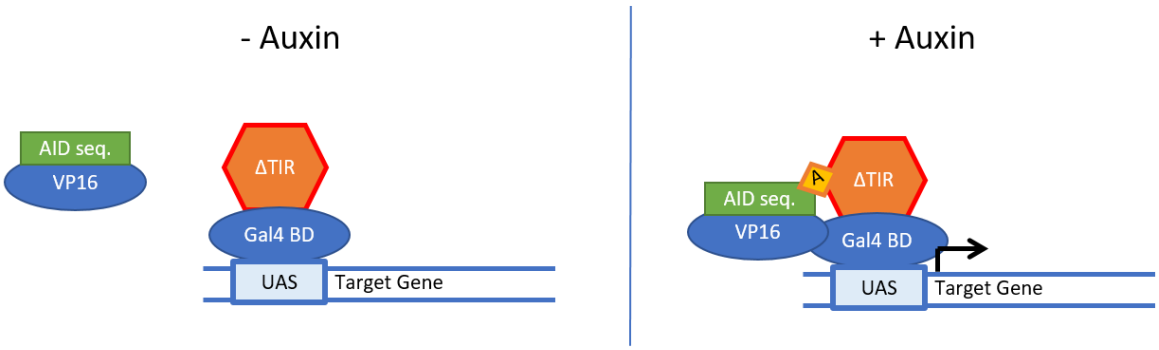


Figure 2.8

This construct is designed to control *in vivo* protein dimerization of a split Gal4 system. The Gal4 binding domain and trans-activator domain are separated from one another. Their dimerization is controlled by the presence of auxin.

-Auxin: VP16-AID and Gal4BD-ΔTIR have low affinity for one another. The Gal4 binding domain still binds to the UAS however the trans-activator VP16 is no present and so there is transcription of target genes.

+Auxin: Auxin (yellow diamond [A]) stabilises binding of AID and ΔTIR. This allows Gal4BD and VP16 to dimerise. Gal4BD binds the UAS and VP16 recruits the transcriptional machinery of the cell allowing for transcription of downstream target genes.

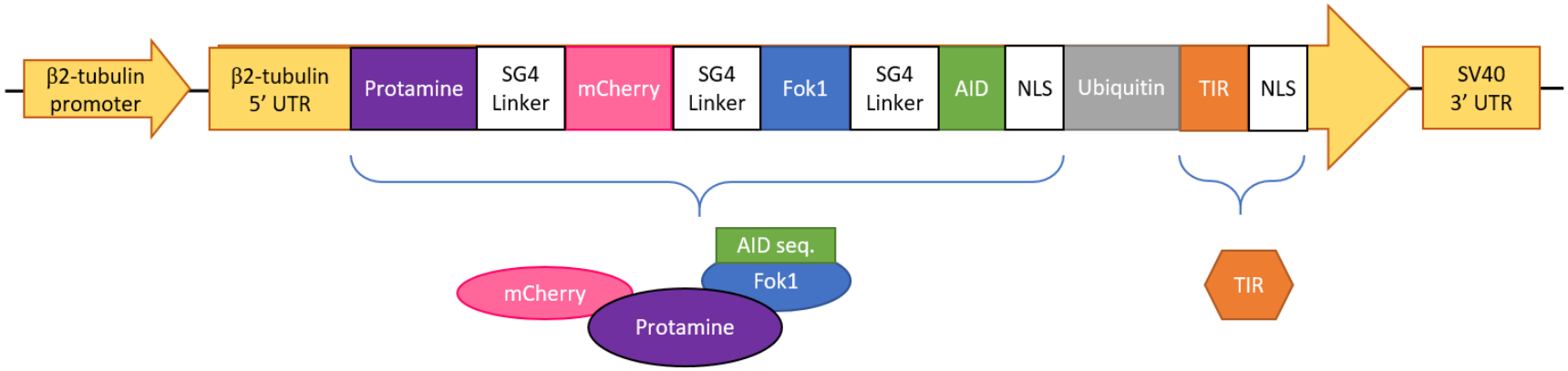


Figure 2.9

Schematic diagram of #4789 (TIR/mCherry-Protamine-Fok1-AID). This construct encodes a single transcript that yields two separate protein products. It is driven by a β2-tubulin promoter that limits expression to male germline. The first is a large fusion protein containing protamine, the red fluorescent protein mCherry, the non-specific nuclease Fok1 and finally the AID tag. Each component is separated by an SG4 amino acid linker sequence that should allow the proteins to fold independently and prevent or limit steric interference. The protamine should ensure that the Fok1 is located at the DNA in late stage spermatids as it replaces histones at this point. mCherry will serve as a marker for the location of the fusion protein. The second protein is TIR.

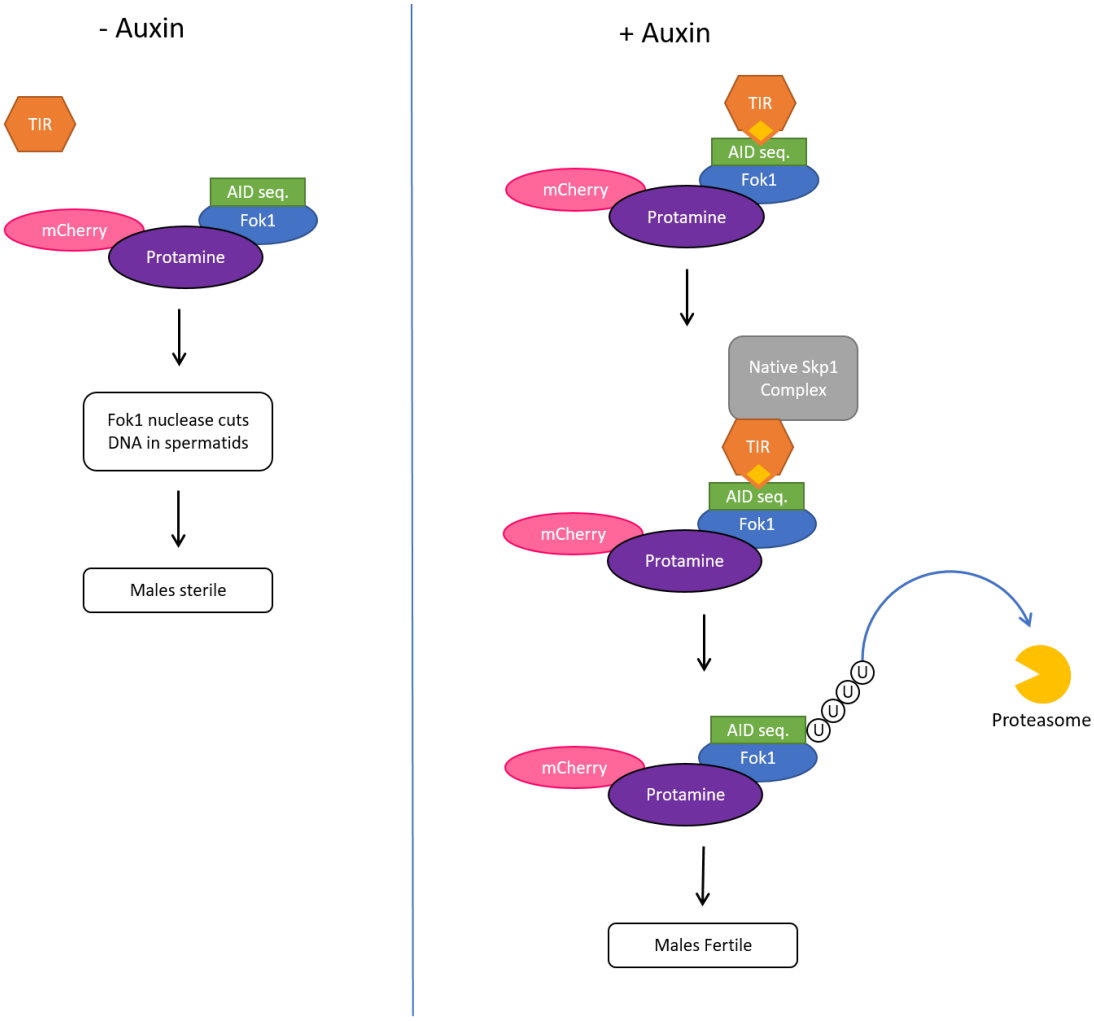


Figure 2.10

An auxin responsive system for controlling male fruit fly sterility.

-Auxin: The AID tag and TIR have low affinity for one another. The Fok1 nuclease is located near the DNA in late stage spermatids. Fok1 makes double stranded breaks in the DNA. This generates males that produce motile sperm that are functionally sterile. **+Auxin:** Auxin (yellow diamond [A]) stabilises the binding between TIR and the AID sequence. The native Skp1 complex of the cell is recruited. This causes the AID sequence to be poly-ubiquitinated. The ubiquitin tail targets the entire mCherry-Protamine-Fok1-AID fusion protein for degradation by the proteasome. Since the Fok1 nuclease is degraded the males are fertile.

2.1.5 Progress Made with Initial Auxin System Constructs

All three constructs were injected by a member of Prof. White-Cooper's research group at Cardiff University. Transgenic lines were established and suitable experimental crosses performed. None of the three designed systems were generating expected phenotypes when reared with auxin in the food.

2.1.6. Initial Aims

Upon joining this research project, the primary aim was to determine whether the constructs were being transcribed and to hypothesise on reasons why the systems were not functioning. Following this the aim was to design and test a simpler bi-partite system that could utilise auxin to induce male sterility.

2.2 Methodology

2.2.1 *Drosophila* Rearing

All constructs were injected into the *w*^{1118} *Drosophila melanogaster* background and crosses were made to this background, originally sourced from Bloomington. The stocks were reared in plastic bottles or tubes containing *Drosophila* food that was produced in-house (standard maize, dextrose, yeast, agar). Flies were incubated at 25^{o}C for optimal life-cycle of 11 days. Bottles were plugged with cotton balls. They were turned over every three weeks to provide fresh food, a clean environment and to ensure that the genotype of the stock was maintained. To sex, screen and sort adult flies, tubes or bottles were tapped out onto a gas pad with constant CO_2 flow and viewed under a binocular microscope. They were then carefully manipulated with a fine paintbrush to prevent damage.

Crosses were performed using virgin females and males that were a few days old as this encourages mating. Newly eclosed females were selected first thing in the morning and can be recognised by their increased size and grey colouring, they do not mate for the first 8 hours providing a window for removal. Older females and were discarded and males were separated, this allowed for more virgins to be collected in the afternoon. Separate male and female tubes were kept with 20 – 30 of each sex. Stocks were starred with 4 – 8 females in a tube and 10 -15 for a bottle although up to three times that amount were used if the stock contained a mutant or subsequent alteration that might impact survival. Once crossed the parents were removed after four days to ensure they were not mistaken for progeny.

2.2.2 Single Fly DNA Extractions

Individual flies were placed in eppendorf tubes and broken up with disposable plastic homogenisers in 50 µl of squishing buffer (10 mM 1M Tris pH8.2, 1mM 0.5M EDTA, 25 mM 5M NaCl, 0.2 µg/ml Proteinase K made up to 100ml with deionised water). This was incubated at 37^{o}C for 30 minutes. The reaction was terminated by incubating at 95^{o}C for 3 minutes to denature the proteinase. The samples were then spun at 13 000 rpm for 5 minutes and stored at -20^{o}C.

2.2.3 Head and Testis Dissections

Flies were immobilised on a CO_2 gas pad, they were then viewed under a dissecting microscope on a flat plastic disc placed on a black background. Heads were dissected using tweezers and placed in individual Eppendorf tubes containing 50 µl of squishing buffer, and stored at -20°C. Testis were dissected from young male flies and transferred into testis Eppendorf tubes containing 50 µl of testis buffer (183mM KCL, 47mM NaCl, 10mM TRIS pH 6.9). Each Eppendorf tube contained 10 testes and was stored at -20°C.

2.2.4 RNA Purification

Heads were homogenised in 50 µl of squishing buffer using disposable plastic homogenisers. Testis were too small to be broken up in this way and so were homogenised by being drawn repeatedly through a needle with a syringe in 50 µl of testis buffer. RNA purification was carried out using the PureLink® RNA Mini KIT (Ambion. Protocol reviwed 21 May 2012), the protocol was followed as per manufacturer's instructions except for the following amendments:

12. Add 25 µl of RNase-free water to the spin cartridge.
13. Incubate at room temperature for 1 minute
14. Centrifuge at 2 minutes at 12,000 x *g*

These three steps were then repeated to yield a final elution volume of 50 µl. RNA was stored at -20°C if not immediately used. Prior to cDNA synthesis RNA was treated with DNase I to remove any gDNA contamination following the following protocol:

1. In a PCR tube 8 µl of RNA were combined with 1 µl of 10X DNase I reaction buffer, 1 µl DNase I (Amp grade, 1 U/µl).
2. Incubate tube for 15 min at room temperature.
3. Deactivate DNase I by addition of 1 µl of 25 mM EDTA
4. Incubate for 10 min at 65°C

The RNA was then immediately converted to cDNA or used in RT-PCR.

2.2.5 cDNA Synthesis

cDNA synthesis was carried out using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen cat no. 18080-051). Using the following reaction mix: 8 µl RNA, 1 µl 50 µM oligo (dT) and 1 µl of 10 mM dNTP mix. This was mixed briefly using a vortex and quickly spun down before continuing with the first incubation step. cDNA was stored at -20°C if not used immediately for RT-PCR.

2.2.6 Gel Electrophoresis

Samples mixed with a loading dye (xylene cyanol in 30% glycerol or Themo scientific 6x Gel Loading Dye) were loaded into gels of varying agarose percentage (1 – 2.5 %) made with TAE (Tris-acetate-EDTA) buffer and ethidium bromide (0.5 µg/ml). Molecular mass of products was estimated using a 10 kb SmartLadder (Eurogentec). Gels were run at either 25 or 50 V.

2.2.7 Reverse Transcription – PCR

cDNA was synthesised as per section 2.2.5. The reaction mix was then set up as follows:

H_2O	10.25 µl
GoTaq Enzyme	0.25 µl
5x Green Buffer	4.0 µl
dNTP mix	0.5 µl
$MgCl_2$	2.0 µl
Forward Primer	1.0 µl
Reverse Primer	1.0 µl
cDNA	1.0 µl
Total Volume	20 µl

Cycling programmes were modified to allow for different annealing temperatures of the primers used and the length of product expected. A typical programme might look as follows:

1.	94°C for 120 seconds	Activation of GoTaq
2.	94°C for 30 seconds	Denature the cDNA
3.	60°C for 30 seconds	Potential annealing temperature
4.	72°C for 45 seconds	Extension
5.	Return to step 2 for 34 more cycles	
6.	72°C for 300 seconds	Final extension
7.	Hold at 4°C	

2.2.8 Quantitative RT-PCR

Reaction mixes were prepared on 96 well PCR plates to a final volume of 20 µl. Each well contained 10 µl of 2X GoTaq® Green Master Mix 2X (Promega), 1 µl of the forward primer, 1 µl of the reverse primer 1 µl of cDNA and 7 µl of deionised H_2O. Any required dilutions of the primers or DNA were performed beforehand. The PCR was then run according to the following programme:

1. 50°C for 2 minutes
2. 95°C for 10 minutes
3. 95°C for 15 seconds
4. 60°C for one minutes
5. 80°C for 2 seconds
6. Plate Read
7. Repeat from step 2 for 44 cycles
8. Calculate melting curve from 65°C – 95°C

Data was then exported into Microsoft Excel for analysis and quantification against a wild-type control the control gene was PP1-96α. Three replicates were run for each sample (gDNA, head and spermatocytes) per line being tested (WT, #4759 and #4789). The three Ct values were averaged and then used to calculate ΔCt and subsequently ΔΔCt. Table 2.1 gives the primer sequences used to detect PP1-96α and TIR transcript.

Table 2.1 Q-RT-PCR Primers

Gene Name	Forward Primer	Reverse Primer
PP1-96α	5'- CTGCTGGCCTACAAGATCAAGTA	5' - TTGAAGCAGTCCGTGAAAGTTTT
TIR	5'- CCTGACCGATGATTTCCAGAC	5' - CTCAGATTCAGGCCGGTCAG

2.2.9 Preparation of Plasmids for Micro-Injection

pGEM®-T vectors (Promega) were transformed into JM109 competent cells (Promega) as described in the manufacturer's technical manual (for use of products A1360, A1380, A3600 and A3610). Ampicillin agar plates were made and stored at 4°C until required for use. Plates and LB medium were made to the manufacturer's instruction. Agar plates contained a final concentration of 100 µg/ml ampicillin. Following incubation of plates overnight individual colonies were selected and stirred gently into falcon tubes containing 1.5 ml of LB broth with ampicillin at a final concentration of 100 µg/ml. Falcon tubes were incubated overnight at 37.5°C with vigorous shaking. Bacterial cells were isolated by centrifugation and the broth was carefully discarded.

Plasmids were first isolated using QIAprep Spin Miniprep Kits (QIAGEN cat no. 27106) per manufacturer's instructions. This yielded a final elution volume of 50 µl. Concentrations of plasmid DNA were measured using a NanoDrop 1000. Minipreps were then digested to determine whether plasmid product was of the correct size. Samples were also sent to be sequenced to check for mutations. #5085 was digested with BamHI and #5076 was digested with EcoRV. The digest mix was incubated for two hours at 37°C and products were then run on an agarose gel and visualised using a 1 kb ladder (NEB) using the following reaction mixes:

	EcoRV	BamHI
Water	14.8 µl	15.0 µl
10x Buffer	2.0 µl	2.0 µl
BSA	0.2 µl	-
Enzyme	1.0 µl	1.0 µl
DNA	2.0 µl	2.0 µl
Total Volume	20 µl	20 µl

Provided the digests were successful and yielded products of the correct size and the sequences matched what we expected a maxiprep was then performed using colonies from the original transformation plate to generate substantive amounts of DNA. Colonies were transferred into conical flasks containing 75 ml of LB broth and ampicillin (100 µg/ml), cultures were incubated overnight at 37°C with vigorous shaking. Maxipreps were performed using an E.N.Z.A® Endo-Free Plasmid Maxi Kit (Cat No. 6926). Maxi-preps were carried out per the manufacturer's instructions for using centrifugation. Following completion of the protocol plasmid concentration was measured using a NanoDrop and then digested as above except that only 1 µl of plasmid DNA was added to each digest.

Injection mixes were made up to 20 µl with a final concentration of 700 ng/ µl for plasmid DNA and 200 ng/ µl of helper DNA. Each injection mix also contained 2 µl of buffer solution. 2 µl of the injection mixture was loaded into the needle prior to injection.

2.2.10 Microinjections

Embryos were collected within one hour of being laid. This maximises the chances of the injected DNA being incorporated into the germ line as this should be prior to cellularisation. Eggs were collected from egg laying pots every 40 minutes. Eggs were either dechorionated using double sided sticky tape or being bathed in bleach. In the case of double-sided sticky tape, the tape was attached to two separate glass slides. Embryos were carefully transferred from the laying pots to the slides using a fine paintbrush. A copper wire (0.5 mm) was placed around the embryos

between the slides to prevent the embryos from being crushed. The sticky slides were pressed together to stick the chorions of the embryos to the slides. The slides were then separated, removing the chorion and exposing the embryos to the air. This allows the embryos to dry out a little prior to injecting and helped to prevent them from bursting. If the chorion was removed using bleach then eggs were submerged in a 50% bleach solution for two minutes using an embryo basket. The eggs were monitored under a microscope to determine when the chorion had dissolved, at this point the eggs were removed from the bleach and washed in deionised water. They were then transferred to an agar plate to dry and to facilitate transfer.

The slides were then viewed under a binocular microscope (Olympus SZ51), embryos were individually transferred to a glued coverslip (22 x 22 mm) using a pair of tweezers. The embryos were orientated so that they would be injected at the posterior end as this is where the germ line forms. The glue fixes the embryo in place for injection. The glue for the coverslips was prepared by placed 10 cm of double-sided sticky tape into a 15 ml tube containing 10 ml of 100% heptane. This mix was placed on a shaker until the adhesive from the tape had completely dissolved. This mixture was carefully pipetted along one side of the coverslip, the heptane evaporates quickly leaving a thin strip of glue.

The coverslips of fixed embryos were placed onto a slide and the embryos were left to dry for ten minutes at which point they were covered with halocarbon oil to prevent further drying. The slides were then transferred to the injection microscope (Nikon Eclipse Ti-S). A joystick (TransferMan NK2, Eppendorf) was used to position the microneedle at the same plane as the embryos. The embryos were then injected with the injection mix. The injection air pressure was provided automatically (Femtojet, Eppendorf) and a compensatory pressure of 70hPa was used to ensure a constant flow of DNA from the needle. This pressure could be altered if it proved to be inadequate and cellular matrix from the embryos was flowing back into the needle.

Once the embryos on a slide had been injected the coverslip was removed from the slide and transferred to a petri dish containing egg-laying mix (an agar containing apple juice), and small yeast balls to attract the hatching larvae.

2.2.11 Survivor Collection and Crosses

Apple juice agar dishes containing injected embryos were placed in an 18°C room inside a humid chamber to continue development. The lower temperature slows development and maximises the chance of survival. The humid chamber comprised a closed plastic box with wetted tissue paper at the bottom and around the petri dishes. This helped maintain humidity and ensure a hydrating environment for the injected embryos. Surviving larvae were collected during the following three days and transferred to food vials. These vials were incubated at 25°C. Once survivors eclosed as adults they were crossed individually to w^{1118} *Drosophila melanogaster* flies. Females w^{1118} were virgins.

2.2.12 Screening Progeny for Transgenics

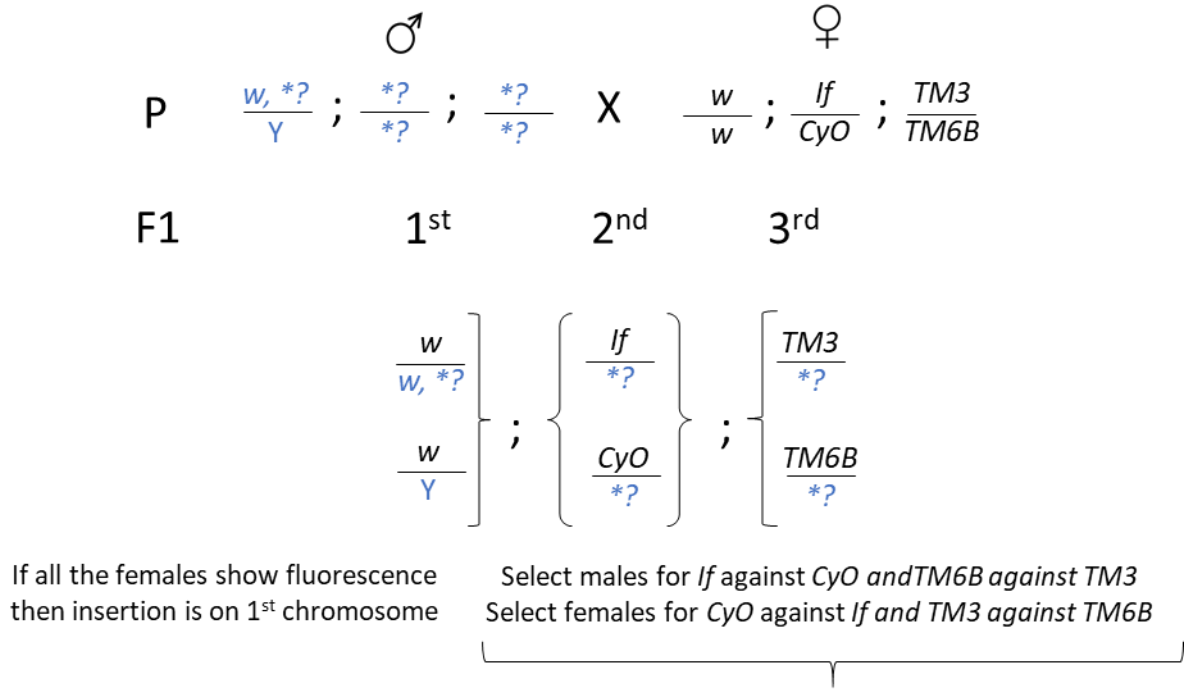
Progeny resulting from survivor crosses were put to sleep on a CO_2 pad and screened using a Leica fluorescence microscope to check for presence of the construct markers. Any resulting transgenics were crossed to w^{1118}*Drosophila melanogaster*.

2.2.13 Insertion Mapping and Balancing of Lines

All balancer stock information was obtained through FlyBase, original stocks were sourced from Bloomington. Transgenic males were selected and crossed to a female carrying balancers on chromosomes 2 and 3 (Figure 2.11). F1 progeny were selected based on phenotype and crossed together. Screening of F2 progeny allowed for the identification of the insertion chromosome. Stocks were then balanced by selecting the appropriate phenotype and crossing back to the balancer population.

2.2.14 Detection of mCherry

Transgenic males carrying Fok1-AID (#5085) were dissected as in 2.2.7 to remove the testis. The testes were carefully crushed on a slide beneath a slide cover with a drop of testis buffer to release the contents for viewing. The buffer was removed slowly with a small piece of blue roll to dry the sample slightly for viewing under the fluorescence microscope. As the testis has been squished it is possible to identify morphologically different cell types including the spermatocytes where we would expect to see expression of mCherry.



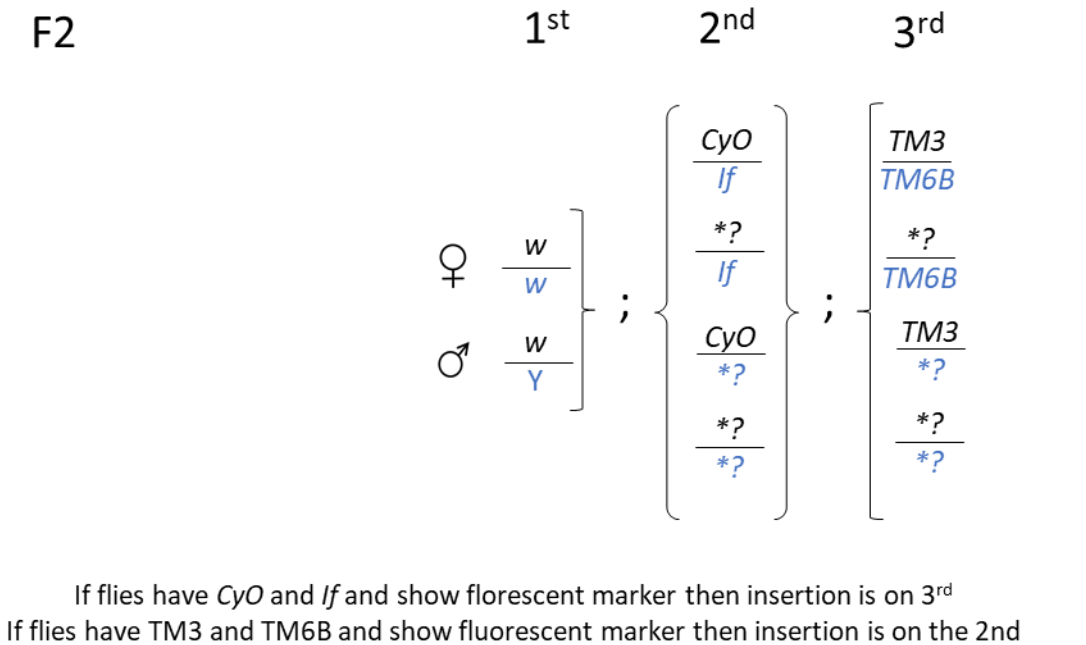


Figure 2.11

Diagram illustrating the mapping process for the insertion (*?). Genes in blue are inherited from the male and in black from the female. Once identified individuals were selected based on their phenotype and crossed back to the balance stock.

2.3 Results

2.3.1 Detection of TIR transcript

Q-RT-PCR was performed to determine the relative levels of TIR transcript present in transgenic *Drosophila melanogaster*. The results were normalised to the expression of the endogenously expressed control gene PP1-96α, to yield the ΔΔCt for Wild-type *Drosophila* and transgenic *Drosophila* carrying either TIR/Gal80-AID (#4759) or TIR/mCherry-Protamine-Fok1-AID (#4789). This gene is expressed at equal levels in all cells and has been used as a successful control in past experiments (Dombrádi *et al.* 1990). Wild type *Drosophila* was set to an expression level of 1 and the transgenic levels were compared to this. PCR was performed on samples of gDNA from transgenic flies to check the primers were able to amplify the target RNA sequences, cDNA purified from a single head to determine if the ubiquitous promoter was functional and finally in testes to check that the transcript is present in the correct region for the system to function as expected.

Table 2.2 below lists the C(t) scores obtained from the Q-RT-PCR. TIR transcript was present in both transgenic lines across tissue type however TIR transcript did not amplify in non-transgenic controls as the C(t) scores are much higher indicating amplification of background sequences instead of the target sequence.

	C(t) value					
	PP1-96a			TIR		
WT gDNA	27.87	28.01	28.5	30.67	32.49	33.4
WT - H	25.43	25.86	26.09	33.34	36.28	32.07
WT - PS	30.37	30.15	29.98	31.85	41.18	N/A
#4759 gDNA	25.12	24.78	25.02	26.77	27.05	26.95
#4759 - H	22.47	23.06	22.86	24.39	24.37	24.17
#4759 -PS	25.64	25.67	25.77	24.04	24.08	24.21
#4789 gDNA	25.41	25.28	25.05	26.44	26.84	26.51
#4789 - H	25.48	24.81	25.26	26.08	25.69	26.48
#4789 - PS	24.08	23.69	23.91	22.57	22.47	22.34

Table 2.2

C(t) values resulting from of a Q-RT-PCR analysis looking for TIR transcript. Read-outs were normalised to the ubiquitously expressed control gene PP1-96α. The primers were run against samples of gDNA extracted from a single male for each line, cDNA extracted from a single head and cDNA extracted from three pairs of testes. Each sample was run in triplicate. WT stands for wild-type background (no transgenes). #4759 and #4789 both carry constructs which should express TIR.

2.3.2 Bi-partite Design for AID responsive system

Due to the nature of the original construct design it was difficult to test individual system components and determine at which point the system was failing to work. To simplify testing, a bi-partite system was designed by myself and Tarig Dafalla (Oxitec. The altered system is similar to another construct (TIR/mCherry-Protamine-Fok1-AID (#4789) as this system does not require crossing with any Gal4 drivers and so has fewer potential failure points. The TIR and mCherry-Protamine-Fok1-AID were placed on separate constructs. Equally the m-Cherry was separated from the rest of the fusion protein by a T2A bridge. There is also an S4G linker between the Fok1 nuclease and the AID sequence. These changes were introduced as a method for limiting steric interference.

The first construct #5076 (β2-tubulinTIR), TIR is expressed in a testis specific manner as it is driven by the testis specific promoter β2-tubulin (Figure 2.12A). The second construct #5085 (Fok1-AID) carries many of the same components from TIR/mCherry-Protamine-Fok1-AID (#4789). The construct is driven by a protamine promoter and should generate two translational products. The first is mCherry this can be used as a marker to determine where the construct is being translated. The second product is a fusion protein comprised of: protamine, to target the nuclease to the DNA; the nuclease Fok1, to cleave the DNA and the AID tag to allow binding to TIR (Figure 2.12B). The β2-tubulinTIR and Fok1-AID constructs were both marked with red eyes (3xP3-dsRed) to allow for identification of transgenic animals.

This system is functionally predicted to work in the same way as TIR/mCherry-Protamine-Fok1-AID (#4789). TIR is expressed in the testis as is the protamine-Fok1-AID fusion protein. These are translated in the spermatid stage. In the absence of auxin TIR does not have a high affinity for the AID sequence, Fok1 is free to cause double strand breaks in the spermatid DNA generating morphologically normal sperm that is functionally sterile. In the presence of auxin, the AID sequence binds to the TIR sequence and targets the Fok1 nuclease for degradation by the proteasome. The males reared on auxin should be fertile (Refer to figure 2.10).

This means that we would expect any male G1 transgenics to be infertile as they should express functional Fok1 which will cleave the spermatid DNA at multiple points. This allows us to determine quickly if the construct is functioning as expected as there should be no progeny resulting from a G0 transgenic male to a wild-type female.

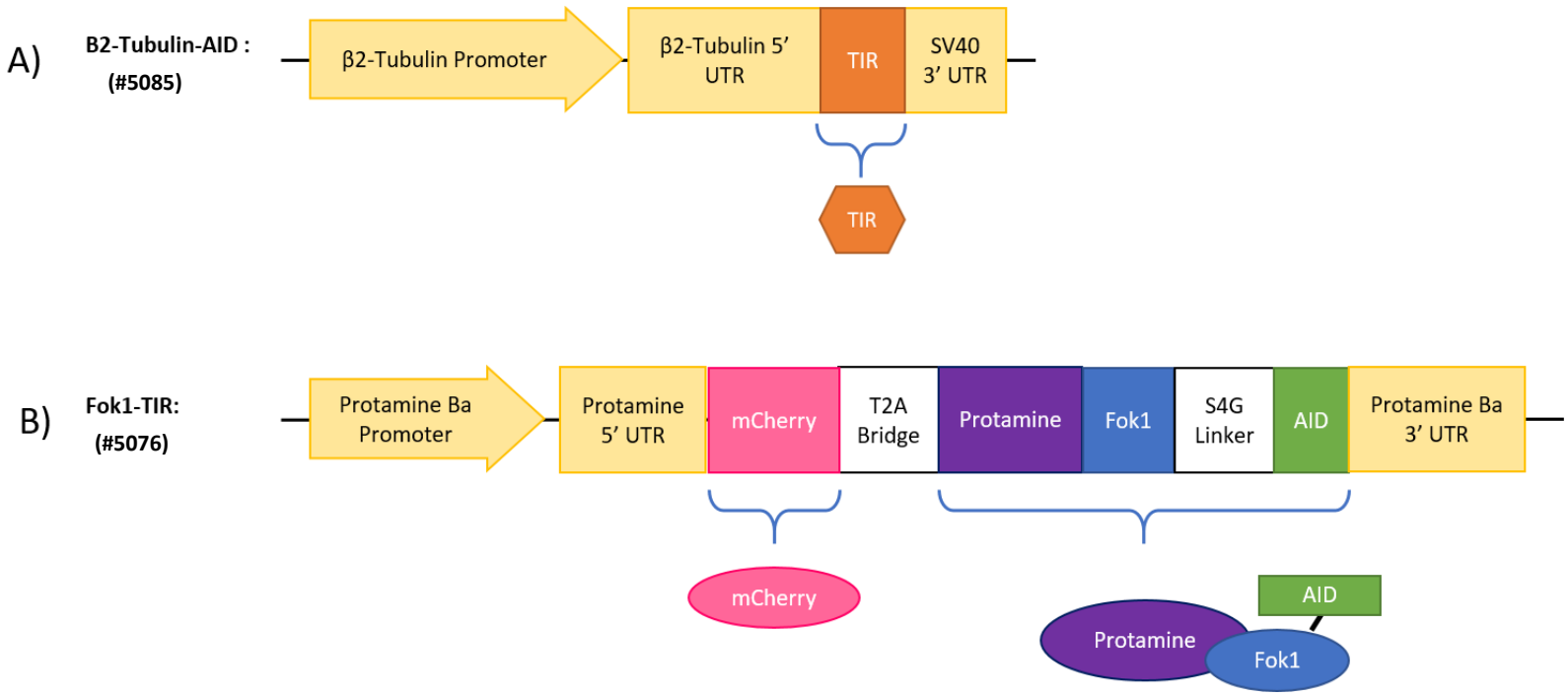


Figure 2.12

A) Construct schematic of β2-Tubulin-TIR (#5076). The promoter ensures that TIR is expressed exclusively in the male germline.

B) Fok1-AID (#5085) construct schematic. The construct is driven by the protamine promoter and generates two protein products. The first is mCherry, during translation the T2A bridge forces a ribosomal skip causing the amino acid chain to dissociate without releasing the mRNA. Translation continues and produces the fusion protein Protamine-Fok1-AID. The Fok1 nuclease is free to cleave DNA meaning that this construct should be dominant male sterile and will need to be maintained in a female line.

2.3.3 Injection of New Constructs and Establishment of Lines

404 embryos were injected with β2-tubulinTIR (#5076), these embryos were dechorionated using the 'sticky-tape' method. In total 114 embryos reached adulthood yielding a 28% survival rate (Table 2.3.1). In total 103 survivors were crossed individually to virgin *w*(118) flies. 24 transgenic lines were initially identified (Transformation rate: 5.9% from starting number injected).

320 embryos were injected with Fok1-AID (#5085). In this case the chorion was removed using a 50% bleach solution. In total 44 injected individuals eclosed, here the overall survival was much lower at 14% (Table 2.3). 41 individuals were crossed to $w^{(1118)}$ virgins. This led to the establishment of 15 lines (Transformation rate: 4.7% from starting number injected).

The G3 progeny were screened and differences were observed in the brightness of DsRed2 expression when comparing siblings. This indicated that there were multiple insertions of some lines. In these cases the different phenotypes were isolated and to capture as many insertion events as possible. After this lines were mapped and balanced. In total 46 lines were established for Fok1-AID (#5085) and 43 lines for β2-tubulinTIR (#5076). It is not known how many of these represent individual insertion events.

Table 2.3
Injection Survival and Transformation Rates for β2Tubulin-TIR (#5076) and Fok1-AID (#5085)

	Construct Name	
	β2Tubulin-TIR (OX5076)	Fok1-AID (OX5085)
Chorion Removal Method	Double-sided tape	Bleach
No. Embryos Injected	404	320
No. Larvae Hatched	129	81
No. Eclosed Adults	114	44
No. Transgenic Adults	24	15
Overall Survival	28%	14%
Transformation Rate	5.9%	4.7%

2.3.5 Sterility Test

For each test a young virgin male carrying Fok1-AID (#5085) was crossed to three virgin w^{1118} females that were 3 – 5 days old and reared on normal fly food. The adults were left for 24 hours and then removed. Any resulting progeny that survived to adulthood were scored. Any male carrying Fok1-AID (#5085) and raised without auxin in the food should be sterile due to the presence of the Fok1 nuclease. To serve as a control one virgin w^{1118} male was crossed to three virgin females. Fifteen individual wild-type crosses were performed to generate a good average. In total 15 different transgenic lines were crossed. All Fok1-AID (#5085) crosses gave rise to progeny indicating that the system does not induce male sterility (Figure 2.13). A one-way ANOVA was performed on the two groups (wild-type and transgenic), there was no significant difference between the means of the two groups ($F_{1,26} = 0.063$, $P = 0.804$).

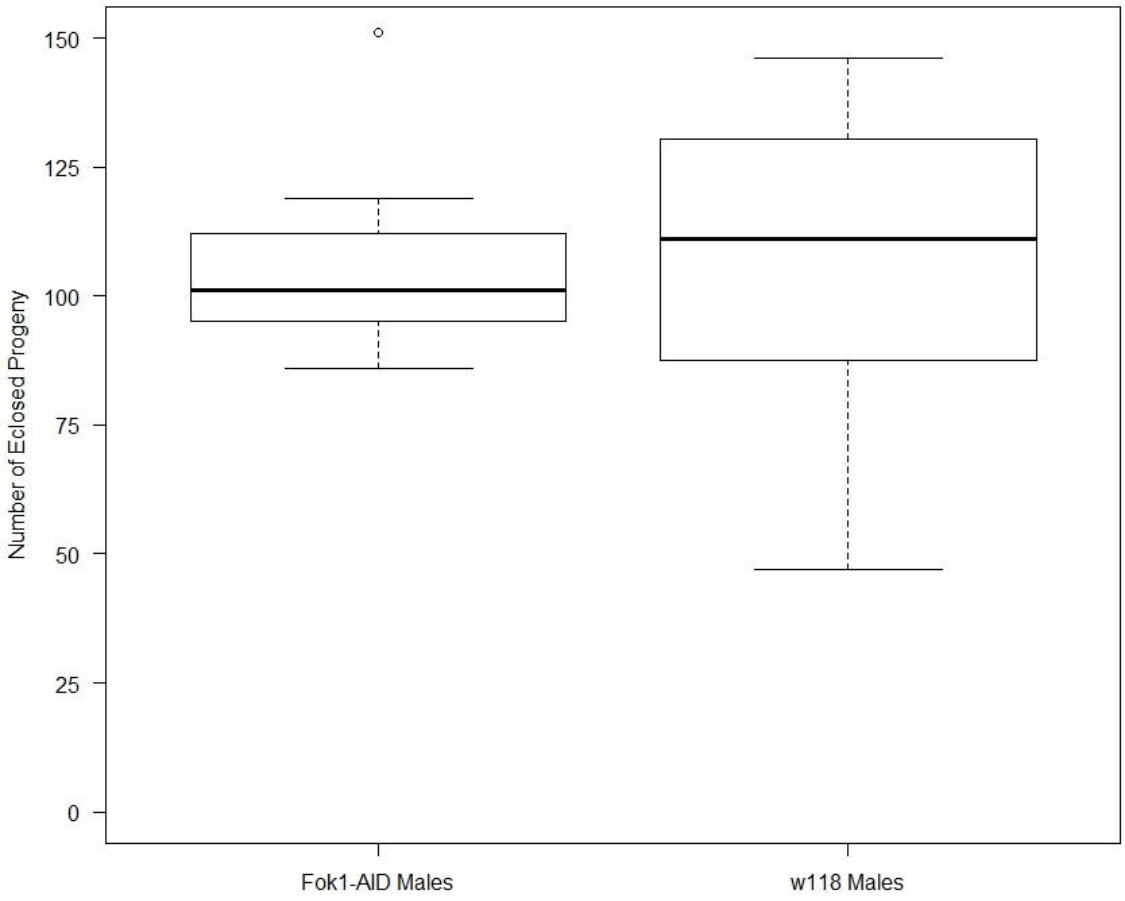


Figure 2.13

The number of progeny that eclosed following a cross between a single male to three females. The single males were either carrying Fok1-AID (#5085) or were w118 (wild-type white eyes background). The Fok1-AID construct is not generating male sterility and therefore is not working properly. The presence of the construct did not reduce the number of offspring compared to a the w118 control (One-way ANOVA: $F_{1,26} = 0.063$, $P = 0.804$)

2.3.5 Detection of mCherry in Spermatids

The testes were dissected from a single young male for each of the 46 lines and visualised under green fluorescent light to determine whether mCherry was present. This would elucidate whether the transcript was being translated and if it is being translated in the correct region at the correct time. Testes were placed on a slide in an excess of testis buffer (183 mM KCL, 47 mM NaCl, 10 mM Tris pH6.9). Pictures were taken of both the phase contrast and the fluorescent image. Six lines in total showed mCherry fluorescence (Figure 2.14) in the spermatids, none of these lines had shown sterility in the fertility test. No lines showed mCherry fluorescence in the primary spermatocyte stage. There is variability in the strength of fluorescence between lines, this is due to the piggybac insertion elements. There is no control over the insertion site and so transgenes are influenced by control elements near the genomic DNA insertion site which will affect expression levels.

In lines where mCherry was visualised and there were enough available males multiple individuals were dissected. There was an inconsistency in the observed mCherry fluorescence between individuals. There was no fluorescence at all in several individuals. Out of three dissected males from the 23I line only two showed fluorescence, with a clear signal detected in spermatid nuclei. Only auto-fluorescence in the sperm was detected in the third individual. This is unexpected as all progeny are from a single female. The males dissected were old (at least 10 days) therefore mCherry in the spermatids may have been degraded in these individuals. It is possible that the female may have had multiple insertions on the same chromosome creating potential for recombination. This may alter expression levels between individuals.

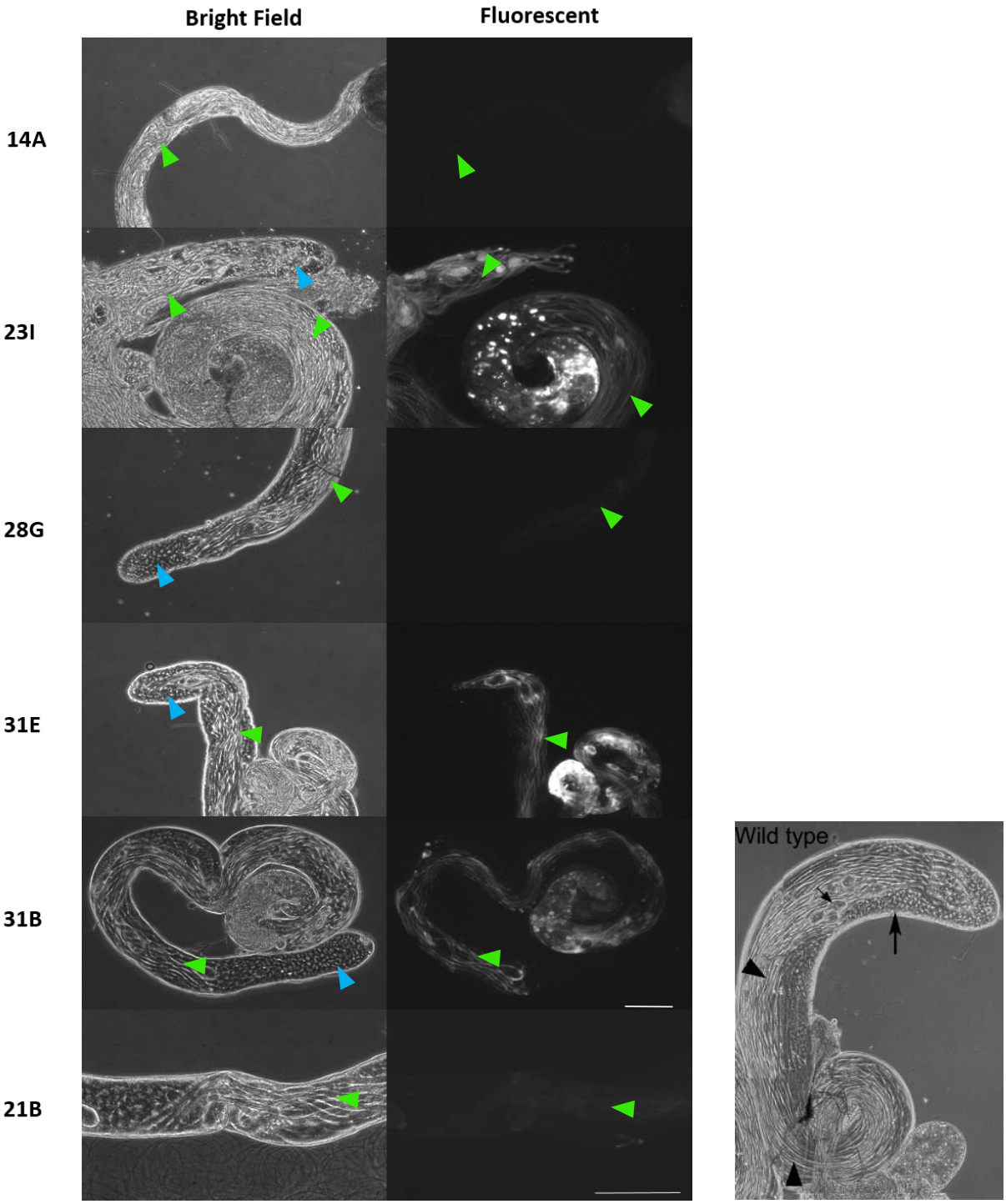


Figure 2.14
Right hand figure: Wild type *Drosophoila* testis viewed under phase contrast viewed after gentle squishing. Several key cell types as indicated. Primary spermatocytes (large black arrow), waste bags (small black arrow) and elongating spermatids (arrowheads) (White-Cooper, 2009).

Left hand figure: Visualisation of mCherry in testis of flies carrying # 5085-AID. In total 6 lines showed mCherry fluorescence in the testis. The signal does not appear to be uniformly distributed along the spermatids (green arrowheads). There is no fluorescence in the primary spermatocytes (blue arrowheads), the transcript is not translated until the spermatid stage. The signal is brighter in lines 23I, 31E and 31B. The images for 21B were taken at 20x magnification the scale bar represents 100 µm. The other five lines were taken at 10x magnification the scale bar represents 100 µm.

2.4 Summary of Outcomes

The intended outcome for this chapter was to engineer an auxin responsive genetic switch in the model insect species *Drosophila melanogaster*. This system would generate male sterility unless reared on food containing auxin. Several stages were completed including:

- The generation of transgenic strains carrying each part of the system, β2-tubulinTIR or Fok1-AID.

- Visualisation of the mCherry marker on the Fok1-AID construct in in the correct location and developmental stage within *Drosophila* testis.

- An initial sterility test between G1 transgenic males carrying Fok1-AID and w118 females that demonstrated the construct was not inducing sterility as intended.

As the Fok1-AID fusion protein was not cleaving spermatid DNA as theorised, crosses to the trans-activator (β2-tubulinTIR) were not performed as we did not have a phenotype to reverse and so auxin responsiveness was not tested.

2.5 Discussion

Transgene control is an essential tool by which researchers can investigate the function of a gene or protein. Conditional knockouts are particularly useful especially when the gene of interest is essential during early development and yields a lethal phenotype if knocked out or down too early. There are several options currently available (discussed in Chapter 1) however as the questions become more complicated more options need to be available to look at gene interaction in more detail.

The auxin responsive system from plants is an attractive system to translate into insect species. The auxin responsive genes are triggered very quickly at very low cellular concentrations. As a plant phytohormone this molecule is not something present metabolically within the insect, the system is also highly specific limiting any potential off target effects. Unpublished data by Ryan Turkel (Cardiff University) showed that auxins are not toxic to *Drosophila* and can be administered safely through feeding.

Although the auxin switch system designed to confer male sterility did not induce male sterility there is at least evidence to show that #5058 (the mCherry-Fok1-AID cassette) was being transcribed in the correct sub-set of cells within the testis. The localisation signals and promoters appear to be working well. There are several potential failure points for this system. The Fok-1 protein may not be folding properly and so is unable to bind and cleave DNA. The transcript could be degraded before it is being translated or before folding.

Since this this project began several papers have been published demonstrating that auxin-dependent degradation of fusion proteins containing the AID sequence is possible and occurs rapidly in a concentration dependent manner (Trost *et al.* 2016; Melinda *et al.* 2017). This system has been transferred successfully to *Drosophila*. They initially tested the system in S2R+ cells with a single construct (pMT-OsTIR1-P2A-H2B-aid-eyfp). The construct yields two products due to the self-cleaving 2A peptide. This feature was present in the original design tested in this work. The products are rice TIR1 and human histone fused to the degron (aid) and yellow fluorescent protein. Again, the sequences used for both the TIR protein and aid are identical to those used in this study. Trost *et al.* (2016) demonstrated that following transfection there was strong expression of the fluorescent protein and that this signal vanished within an hour of auxin exposure, demonstrating that an AID tagged protein could be depleted by recruiting native Skp1 machinery present within *Drosophila* cell lines in the presence of auxin (Trost *et al.* 2016).

This initial work was followed by generating transgenic *Drosophila* lines. They maintained the system in two separate lines. The first *UASt-OsTIR1* has TIR1 downstream from the five tandem

Gal4 binding sites and the hsp70 minimal promoter, TIR1 expression is thus dependent upon the presence of Gal4. The second construct *UASt-aid-rux* generates Gal4 dependent expression of the fusion protein aid-rux which is the auxin inducible degron fused to Roughex. *rux* overexpression interferes with normal cell proliferation. Individuals that expressed *UASt-aid-rux* had significant developmental abnormalities when combined with several tissue-specific Gal4 drivers. Males carrying both *UASt-aid-rux* and *UASt-OsTIR1* were crossed to virgin females carrying the Gal4 driver lines *en-Gal4*. Eggs from this cross were collected and reared in vials either containing 1 mM auxin or not containing auxin. They found that no adults eclosed when auxin was absent however there was some rescue when auxin was present indicating that the aid-TIR1 interaction is working to an extent and there is degradation of the rux fusion protein. The system was not able to fully silence rux activity as adults that eclosed reared with auxin had wing abnormalities (Trost *et al.* 2016).

Bence *et al.* (2017) additionally demonstrated that the AID system can be employed during oogenesis enabling rapid and reversible protein degradation in the ovary. Moreover, overexpression of TIR1 did not influence viability or fertility in adult flies. They also successfully combined the AID system with CRISPR/Cas9-based genome editing to eliminate an endogenous protein. Using CRISPR/Cas9 homologous recombination they inserted the AID motif before the stop codon of the *vasa* gene. Flies carrying AID tagged *vasa* were crossed to flies expressing TIR1 in a Gal4 specific manner. vasa degradation was tissue-specific and reversible in the presence of auxin.

These initial studies demonstrate that the AID system is functional in *Drosophila* and can be combined with existing Gal4 reporter and driver lines to place genes of interest under the control of the small molecule auxin. The system has plenty of scope for development as there are currently no published studies demonstrating auxin-induced protein dimerization or auxin-mediated Gal80 degradation.

Chapter 3: Towards a Vanillic Acid Switch in *Aedes aegypti*

3.1 Introduction

3.1.1 The Lifecycle of *Aedes* mosquitoes

The lifecycle of *Aedes aegypti* has both a terrestrial and aquatic phase involving a complete metamorphosis (Figure 3.1). Eggs hatch around 48 hours after being laid, once embryonic development is complete. This process can take up to five days, depending upon environmental conditions. Once hatched, larvae feed on micro-organisms in their environment, shedding their cuticle to progress through four larval instars. They breathe by resting vertically near the surface and drawing air in through a siphon. After they have amassed enough reserves they metamorphose into an aquatic pupal stage. Larval development is faster in males, so they pupate before the females (Christophers 1960).

At the pupal stage, there is considerable sexual dimorphism: the females are considerably bigger than the male pupae, there is also a morphological difference at the base of the tail (Figure 3.2), making sorting of the sexes easy. The developmental time between hatching and pupation varies depending on temperature, larval density and food availability, under optimum conditions this takes a week but can take up to two. The pupae have no mouthparts but react to sudden changes in light and are capable of movement by thrashing their tails. Pupae breathe from ear trumpets present on the top of the head and can only do so whilst resting at the water's surface. Inside the pupal body the adult is forming fully (Christophers 1960; Nelson 1986).

The adult emerges from the pupal case head first on the surface of the water; males emerge before females. Once emerged, adults mate on the wing, and can survive for up to a month, dependent upon environmental conditions. The males recognise the females by the frequency of their wing vibrations (Maritime 2010). Once mated, a female can store the sperm in spermathecae and use it to fertilise all the eggs she will produce in her lifetime (Christophers 1960).

Before the eggs can be produced the female must first imbibe a blood meal. Both sexes are attracted to the same vertebrate host even though only the females require a blood meal. *A. aegypti* preferentially feed upon humans, they bite multiple times, probing for a good site. The blood meal provides essential proteins for the formation of eggs. A female can lay up to 500 eggs in her lifetime in several clutches and requires a blood meal for each clutch. These clutches are often split between several different sites (WHO 2009).

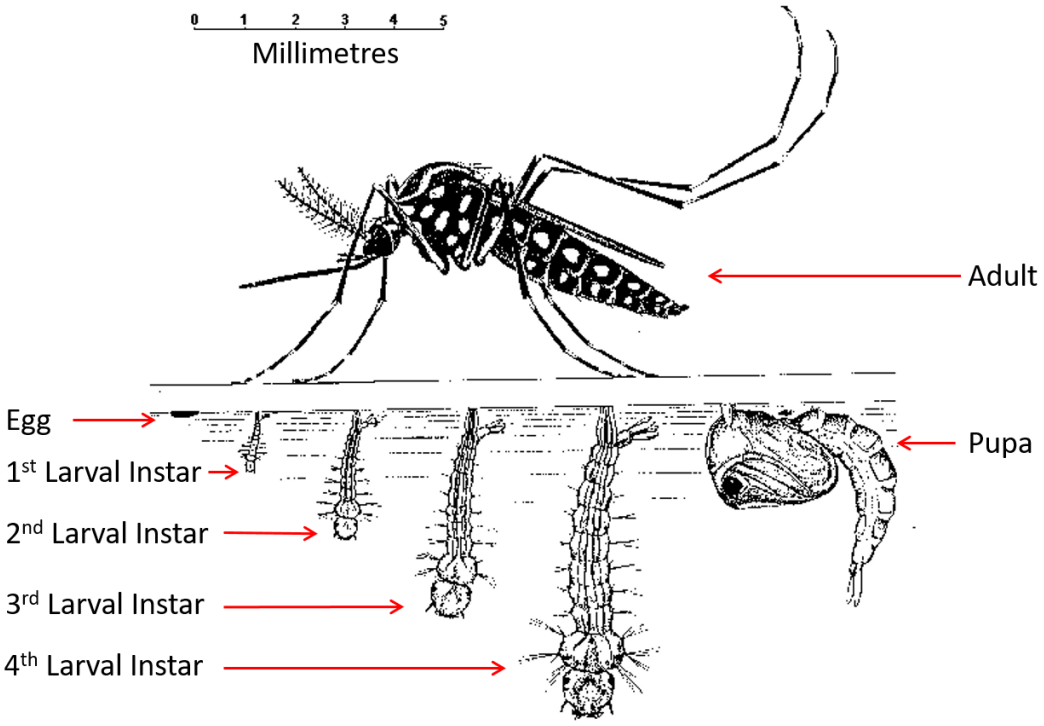


Figure 3.1

The lifecycle of *Aedes aegypti.* It has an aquatic juvenile and a terrestrial adult phase.

Adapted from: https://dengue.cecom.unicamp.br/?page_id=215

[Accessed 02 FEB 2017]

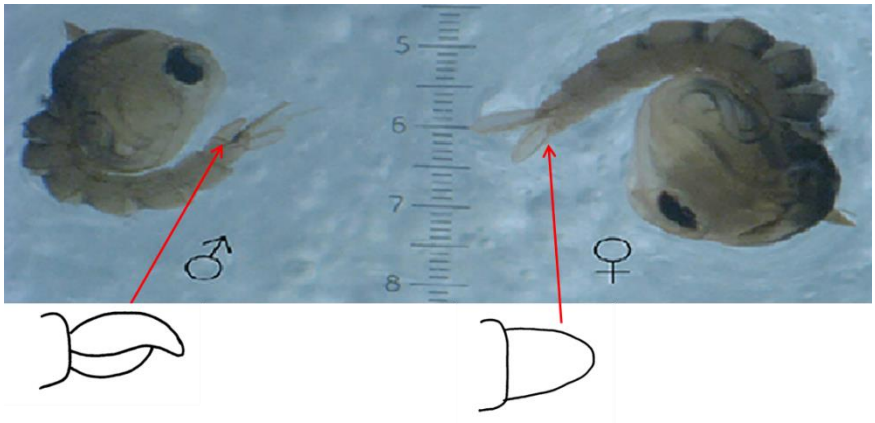


Figure 3.2

Sexual dimorphism between *Aedes aegypti* pupae. The males are significantly smaller than the females they also have differing morphology in the terminal body segment.

Adapted from:

https://www.researchgate.net/figure/259766556_fig5_Figure-7-Distinguishing-male-and-female-Ae-aegypti-pupae-Ae-aegypti-pupae-can-be

[Accessed 02 FEB 2017]

Females choose a variety of locations in which to lay their eggs, some naturally occurring but more commonly in human-made sites that have filled with rainwater (Figure 3.3). Eggs are laid just below the surface of the water; they are oval and around a millimetre in length. The eggs are soft and white initially but quickly harden and turn black. Once embryogenesis is complete the eggs can survive in a desiccated state for months at a time (WHO 2009; Christophers 1960; Nelson 1986).

This facilitates their dispersion; eggs are frequently laid in ephemeral pools where water levels are not likely to be constant. Once the container is re-filled the eggs are stimulated to hatch when bacteria lower the oxygen tension in the water. This means that the eggs can be carried by car, plane or ship to a host of other environments where the eggs can then hatch and establish a new population (WHO 2009; Nelson 1986).

Figure 3.3

Potential egg laying sites for female *Aedes aegypti*. A: Natural environment, rainfilled tree cavity, B: Large discarded items, C: Small discarded items, D: Animal feed bin, E and F: Abandoned or broken sceptic tanks

Images taken from: https://www.cdc.gov/dengue/entomologyecology/m_habitats.html [Accessed 02 FEB 2017]

3.1.2. *Aedes* Mosquitoes as Vectors for Disease

The mosquito species *Aedes aegypti* and the closely related *Aedes albopictus* are the primary disease vectors for several established and emerging arboviruses including dengue, chikungunya, yellow fever and zika (SAGE and WHO secretariat 2016).

Aedes aegypti is distributed throughout the tropics and sub-tropics with particularly high occurrences in Brazil, South East Asia, South America and Africa. Its predicted range is expanding to include areas of Europe (Greece and Spain) and temperate areas of North America particularly in the state of Florida. *Aedes albopictus* is predicted to have a similar range except that it appears to be more suited to Mediterranean and North American temperate zones (Figure 3.4) (Kraemer *et al.* 2015).

Disease transmission occurs during the adult terrestrial stage and is perpetuated by the females. A blood meal is required for egg production, when females feed from an infected individual the virus is transferred from the human into the mosquito. The virus is incubated inside the female until she feeds again, transferring the virus to a new human. Disease transmission is therefore closely linked to female mosquito longevity, the longer an adult female survives the more blood meals she requires and the more people she has the potential to infect.

Dengue is a mosquito-borne viral infection that historically existed as four distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) (SAGE and WHO secretariat 2016) until the recent isolation of a fifth serotype (DENV-5) (Mustafa *et al.* 2015). It is a single stranded RNA virus belonging to the *Flavivirus* genus. In most cases the disease causes flu-like symptoms however in a minority of cases it progresses to dengue haemorrhagic fever or dengue shock syndrome. The more severe cases of dengue can prove fatal although with prompt medical intervention the likelihood of fatality is less than 1%. Exposure to one serotype leads to lifelong immunity for that serotype but only provides temporary cross-immunity to the other three. Subsequent infections by a different serotype increases the severity of the disease (Halstead 1988; WHO 2009; SAGE and WHO secretariat 2016). Current estimates suggest that 4 billion people are at risk of contracting dengue fever and around 390 million cases are contracted each year (Bhatt *et al.* 2013). This estimate is conservative and is likely to be much higher as there is a lack of reporting and potentially a high proportion of misdiagnosis. The number of people infected with dengue represent an enormous burden to healthcare systems in terms of infrastructure and healthcare costs (SAGE and WHO secretariat 2016). A study on the annual economic burden of dengue to 12 countries in SE Asia estimated a total cost of US$950 million per annum, this total did not include expenditure on vector control strategies or other prevention methods (Shepard *et al.* 2013).

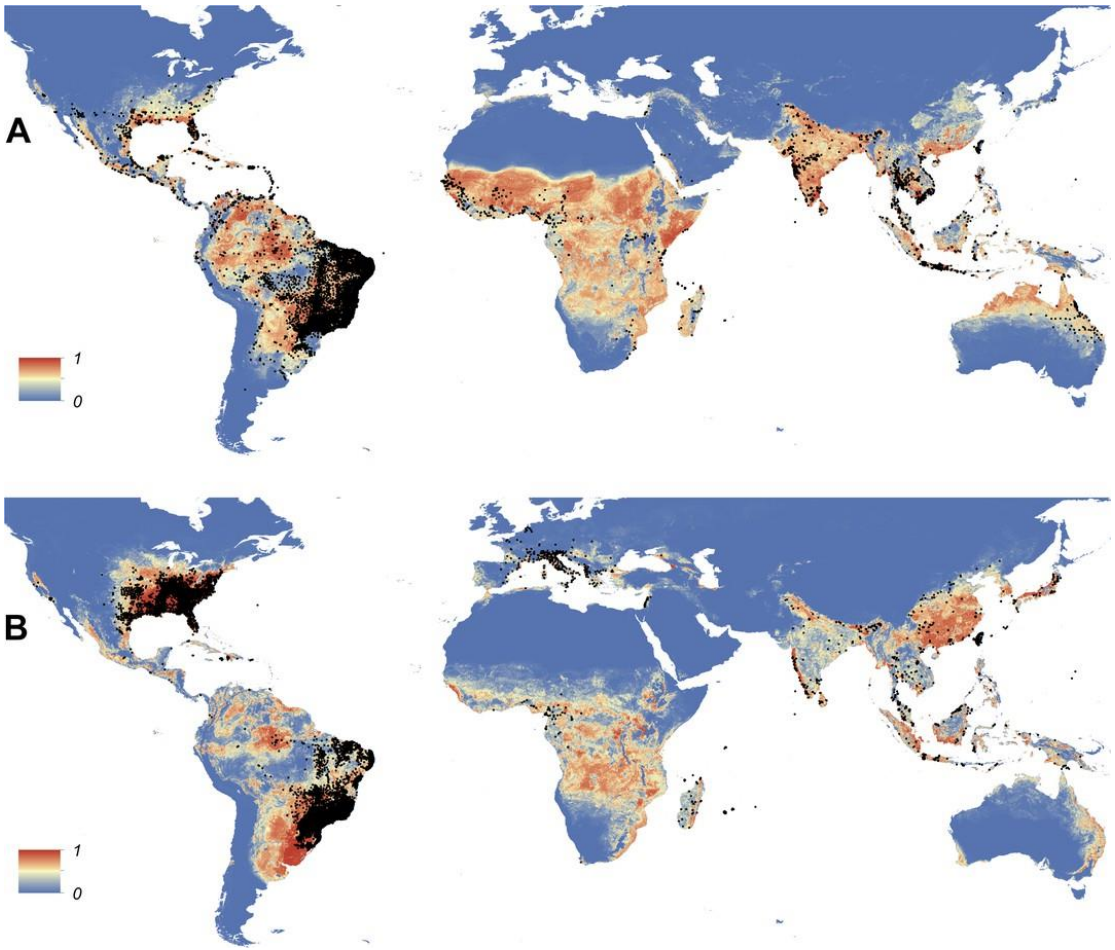


Figure 3.4

The predicted geographical range of *Aedes aegypti* (A) and *Aedes albopictus* (B) with the recorded occurrence of the species shown in black. Blue areas represent no chance of population establishment (P = 0) whilst red areas are guaranteed population centres (P = 1).

Figure taken from Kraemer *et al.* (2015) https://elifesciences.org/articles/08347/figures [Accessed 05 JUL 2017]

Zika is another *Flavivirus* transmitted by *Aedes* mosquitoes. It was first reported in monkey populations in Uganda in 1947. The first recorded human infections occurred in Uganda and the United Republic of Tanzania in 1952. However, the first outbreak occurred 55 years later in Micronesia with 49 confirmed cases, prior to this there had only been 14 documented cases in humans globally. Following this there was an outbreak in Brazil with an estimated 440,000 – 1,300,000 cases in 2015 (Wikan and Smith 2016). Since then there have been documented cases in over 60 countries (CDC 2017). Although the disease itself is not life-threatening in adults, causing mild fever and skin rashes, there is an association between pregnant mothers infected with the disease and the incidence of microcephaly and Guillain-Barré syndrome in new born infants (Wikan and Smith 2016; Cao-lormeau *et al.* 2017; Stigler *et al.* 2015).

Chikungunya, another emergent arbovirus of the family Togaviridae, is also spread by *Aedes aegypti* and *Aedes albopictus.* The name for the disease is derived from the Kimakonde language and means “to become contorted” due to the stooped appearance of sufferers, caused by severe and debilitating joint pain. As with Zika this virus was identified in 1952 in the United Republic of Tanzania. Historically the disease was predominantly limited to Africa, Asia and the Indian subcontinent (WHO 2017). In 2007 there was a localised outbreak in Italy with 197 confirmed cases, confirming that the disease could be spread within Europe (Rezza *et al.* 2007). As of 2016 the CDC had confirmed local transmission of the disease in 45 countries in the Americas and over 100 countries globally (CDC 2016).

Currently treatment methods focus on prophylaxis and the alleviation of symptoms and there are no commercially available drugs for the specific treatment of dengue, zika or chikungunya. There are currently no licensed vaccines for zika or chikungunya. In 2015 the first tetravalent vaccine was licensed for use in Mexico in 2015 called Dengvaxia® (CYD-TDV). Whilst this represents huge strides in the fight against Dengue the drug has variable effectives dependent upon age, previous exposure and severity of infection. Due to the paucity of medical interventions most disease control centres advise focussing efforts on vector control as a means for limiting the spread of disease (SAGE and WHO secretariat 2016).

3.1.3 Traditional Vector Control Strategies for *Aedes aegypti*

As transmission is dependent upon the population density of the *Aedes* mosquito then limiting population size should limit disease incidence. Control methods can target the immature aquatic stages (larvae and pupae) or the adult mosquito directly. The insects are extremely anthropophilic feeding and ovipositing inside homes in water storage drums, overhead tanks, bottles, buckets and discarded items filled with rainwater (WHO 2009; SAGE and WHO secretariat 2016).

Vector control strategies have been employed to target all stages of the mosquito life cycle from egg to adult mosquito. Currently employed strategies target the organism directly through insecticides and natural predator introduction, or target oviposition sites through sanitation, environmental or structural improvements (Achee *et al.* 2015).

Insecticides are the most commonly employed direct method for *Aedes* control. Application focusses on indoor residual spraying (IRS) and peridomestic spraying. Whilst these can prove to be effective methods for reducing numbers of *Aedes aegypti* on a very local level and potentially eradicate the mosquito from a treatment area (Paredes-esquivel *et al.* 2016), there is no proven link that insecticide fogging or residual spraying reduces disease incidence despite reducing mosquito numbers (Bowman *et al.* 2016). This is mostly likely because it is also exceptionally difficult to remove or destroy all the eggs within an environment as a result peridomestic spraying does not reduce population sizes for long time periods. Therefore, there is not a sufficient dip in mosquito numbers to affect disease incidence. In addition to logistical challenges (expenses, staff training, ensuring all areas are adequately treated), *Aedes aegypti* has demonstrated resistance to the four major classes of insecticide (organophosphates, organochlorines, carbamates and pyrethroids) limiting their use in future control programmes (Vazquez-prokopec *et al.* 2017; Vontas *et al.* 2012; Ranson *et al.* 2008).

3.1.4 Genetic Vector Control Strategies

3.1.4.1 The Sterile Insect Technique (SIT)

The sterile insect technique (SIT) involves rearing and sexually sterilising large numbers of the target species and releasing them into the wild. The sterilised males mate with wild females and resulting eggs are non-viable. This causes the population to decline (Klassen and Curtis 2005).

The first field trial of this technique involved two species of tsetse fly and was performed by Vanderplank (1947). Sterility was induced through hybridisation of *Glossina swynnertoni* and *Glossina mortisans*, hybrid offspring from this pairing are semi-sterile. By releasing large numbers of *G. mortisans* into an area populated by *G. swynnertoni* there were enough hybrid matings to eliminate the *G. swynnertoni* population. Vanderplank correctly hypothesised that the *G. mortisans* population would perish due to unfavourable climatic conditions. This represents the first use of genetics to control a wild insect species (Vanderplank 1947).

The technique was further developed through the 1950s in most cases hybridisation as a method of sterilisation was replaced by subjecting insects to low levels of ionising radiation. SIT programs involving this method of sterilisation have had great success at eradicating and controlling several insect species including New World Screwworm, Mediterranean fruit fly and the codling moth (Krafsur 1998; Klassen and Curtis 2005).

SIT programs have been conducted on mosquito species by employing a variety of sterilisation techniques including: gamma radiation, chemo sterilisation, hybridisation and cytoplasmic incompatibility. These programs have met with limited success or failed in most cases, however, cytoplasmic incompatibility was successfully used as a sterilisation method to eradicate *Culex quinquefasciatus* from Okpo, Myanmar in 1967 (Benedict and Robinson 2003). A recent field trial in Italy released *Aedes albopictus* males subjected to low level gamma radiation and saw a reduction in population numbers (Bellini *et al.* 2013).

SIT has many favourable qualities in terms of vector control. It relies on the mate searching behaviour of the male it is targeted and species specific. It is also reversible as populations have the potential to recover by ceasing releases of sterilised males, this limits environmental impacts (Alphey *et al.* 2010; Krafsur 1998).

In order to be successful SIT programs need to produce extremely large quantities of sexually active, sterile insects that can be released over a large area. These programs have several inherent problems that result in their failure or reduced effectiveness. Primarily the mode of sterilisation causes dramatic reductions in competitiveness for females and reduces adult life span. This increases the numbers of sterilised insects required to have a noticeable effect on the

population. In addition, releases should be male only as released females would frustrate mating efforts of the sterilised males and in the case of mosquitoes the females are the disease vector and so releasing females could increase disease incidence. This adds an additional step to the process where males must be separated from females prior to release. In some cases, this can be done mechanically based on pupal size or time of eclosion however these processes can potentially damage the male further (Alphey *et al.* 2010; Benedict and Robinson 2003).

These problems have led to the development of advanced genetic based systems to improve the effectiveness of SIT based programs. These technologies exploit the mate seeking behaviour of males to deliver a genetic mechanism that is passed onto subsequent generations to instigate a population wide change. These new technologies employ a variety of methods including: gene drive systems to distort wild sex ratios, release of insects carrying endosymbionts that introduce refractoriness to disease and self-limiting genes to directly reduce population size (Harvey-Samuel *et al*, 2017).

3.1.4.2 Sex Ratio Distorting Technologies

Engineered gene drive systems rely on the use of naturally occurring 'selfish' genetic elements that distort traditional mendelian ratios to increase their prevalence within a population (Burt 2003). Homing endonuclease genes (HEGs) are selfish gene elements that encode a sequence specific endonuclease. The recognition sequence for the HEG typically occurs once in the genome. The HEG itself is located within the recognition site ensuring that only chromosomes that do not contain the HEG are cut as they will have the completed recognition sequence. In a heterozygous cell the homolog that lacks the HEG is cleaved, this activates the recombinational repair system of the cell and the break is repaired by copying the HEG positive homolog. This results in a cell that contains two copies of the HEG (Figure 3.5). This process ensures that in a heterozygous pairing the HEG will be passed on to 70%, 90% or 99% of the offspring, depending on the efficiency of the system, rather than the expected mendelian ratio. Quickly spreading the HEG through a population (Burt and Koufopanou 2004). In theory HEGs can be engineered in insect species to spread specific genes through a wild population (Burt 2003).

The HEG *I-PpoI* was first inserted onto the X chromosome of *Anopheles gambiae* if this gene were to be expressed during spermatogenesis then the X chromosome would be cleaved incapacitating it and altering the sex ratio of the offspring. This was first tested in *A. gambiae* using the β2-tublin promoter to target the testis. Males carrying β2-tubulin – I-PpoI produced only spermatids carrying Y chromosomes as the X chromosomes were shredded by I-PpoI. Unfortunately crosses to WT females resulted in dominant lethality as the I-PpoI was still active after fertilisation resulting in cleavage of the maternal X chromosome (Windbichler *et al.* 2008). Further work by Galizi *et al.* (2014) resulted in an I-PpoI endonuclease that was destabilised to reduce its half-life and limit its active period to male meiosis, ensuring the protein is not active after fertilisation. They successfully generated transgenic males whose progeny were >95% male. These transgenics induced a population crash in caged trials. Unfortunately, this method cannot readily be applied to *A. aegypti* as this species lacks separate sex determining chromosomes.

Sex determination in *Aedes aegypti* is mediated by a dominant male factor called the M factor which is located within a Y chromosome like region called the M locus. The M locus is rich in repeats making it difficult to identify the M factor genes. Hall *et al.* (2016) characterized *Nix*, a distant homolog of *transformer-2*, which is involved in splicing sex determination regulators in *Drosophila melanogaster*. Female mosquitoes that expressed *Nix* ectopically showed masculinization or deformities of the genitalia. *Nix* is therefore a potential target for genetic vector control through distorting the sex ratio of wild populations.

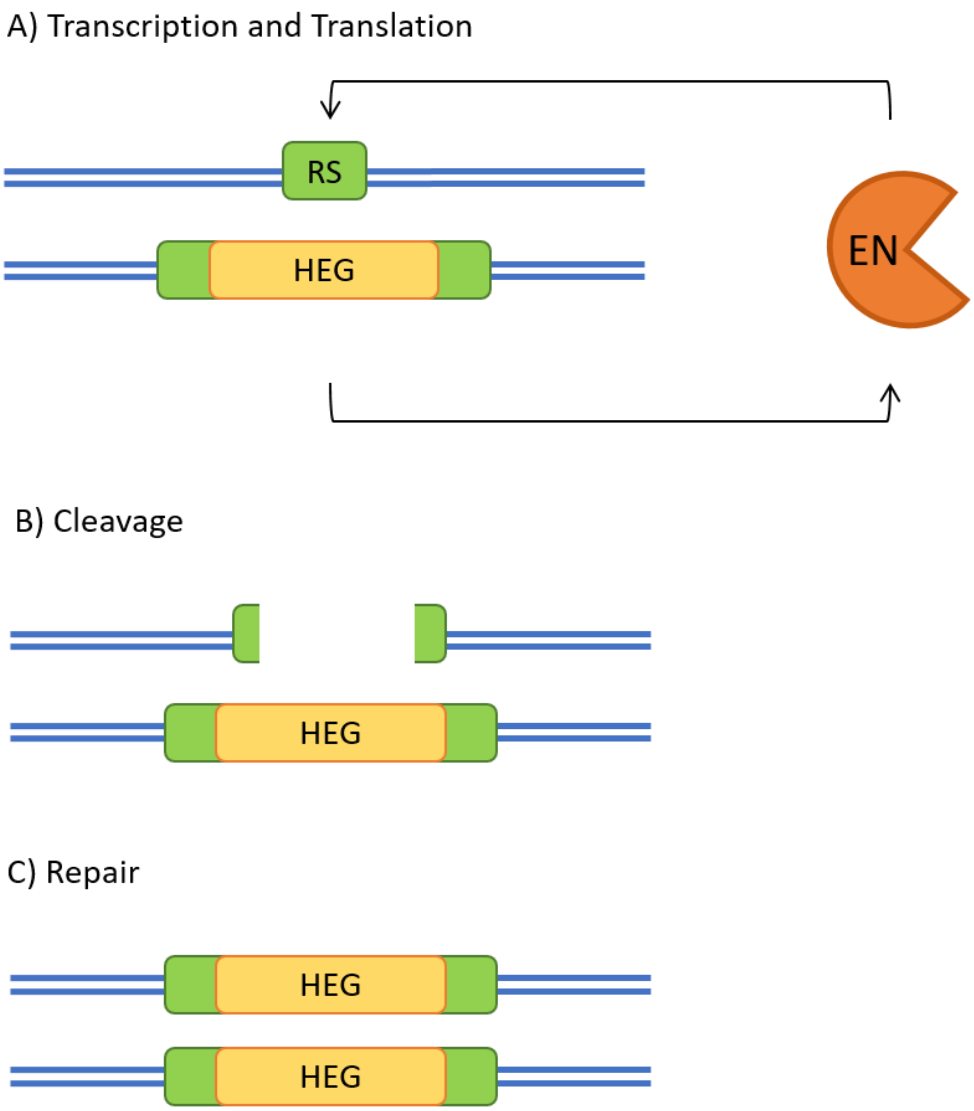


Figure 3.5

Method of action for homing endonuclease genes (HEGs). A) HEGs encode an endonuclease protein (EN) that cleaves a specific recognition site (RS). B) The EN cleaves the RS however the homolog containing the HEG is protected as it is located within the recognition site, disrupting it. C) The cells repair system is activated using the homolog containing the HEG as a template. This leaves a cell that is homologous for the HEG.

Adapted from: Burt and Koufopanou (2004)

3.1.4.3 Increased Refractoriness to Disease

Wolbachia pipientis is an endosymbiotic bacterium that manipulates the reproductive biology of the host to ensure it has a transmission advantage in host populations (Alphey 2014; Sinkins and Gould 2006). The most understood mechanism for how this is achieved is cytoplasmic incompatibility (CI) (O'Neill and Karr 1990; Breeuwer and Werren 1990). The bacterium is transmitted vertically, from mother to offspring, through the egg cytoplasm. When a *Wolbachia* infected male mates with an uninfected female there are no viable offspring however infected females can successfully mate with both infected and uninfected females and give rise to infected progeny (Figure 3.6). This ensures that the bacterium spreads through the population until it reaches fixation, this is termed unidirectional CI (Turelli and Hoffmann 1991).

When there are two or more *Wolbachia* strains present in the population there is CI between each of the strains. Therefore, any matings between individuals carrying different strains do not give rise to any progeny. In this case, whichever strain achieves the majority first will continue through to fixation and the other strains will go extinct in the population. This is known as bi-directional incompatibility (Figure 3.6). However, if bi-directionally incompatible *Wolbachia* strains are co-infected in the same individual to form a superinfection then inheritance in the offspring follows that of unidirectional CI. This means that females carrying both strains can mate successfully with males of any infection status however superinfected males will only mate successfully with superinfected females (Figure 3.7). Through this mechanism the superinfections should spread to fixation in populations that only harbour one of the strains. This system of biased inheritance could be used to artificially drive a preferred trait into a population (Sinkins *et al.* 1995).

*w*MelPop, a *Wolbachia* strain originally found in *Drosophila melanogaster*, significantly reduces host life span (Min and Benzer 1997) and if spread within a mosquito population this would have a significant impact on disease transmission. Adult females will have less time to blood feed and oviposit limiting their reproductive potential (Sinkins and Gould 2006). *Aedes aegypti* transinfected with Drosophila *Wolbachia* strain *w*MelPop-CLA showed a shortened life span and increased refractoriness to disease. The bacterium has been shown to limit the pathogen replication of dengue, yellow fever, chikungunya (Moreira *et al.* 2009) and zika virus (Aliota, Peinado, *et al.* 2016a) within the host. The full mechanism for how this is achieved is still not fully understood however work by Pan *et al.* (2012) determined through micro-array analysis that many genes with functions relating to immunity were up-regulated in *A. aegypti* infected with *Wolbachia* in particular immune effector genes that are linked to the Toll pathway which is an essential immune response pathway that leads to the activation of anti-microbial peptides. An alternate theory is that there is direct competition for resources between the *Wolbachia* and

viral infections within the cell. The two infections co-locate within the insect supporting this theory (Moreira *et al.* 2009).

Unfortunately, the *w*MelPop-CLA strain has significant impacts on fitness (Turley *et al.* 2009; Yeap *et al.* 2011; Ross *et al.* 2014). There have been several field trials however the number of infected individuals at these sites decreased once releases had stopped rather than continuing to fixation (Nguyen *et al.* 2015). Another promising *Wolbachia* candidate is *w*Mel, again from *D. melanogaster*. Mosquitoes carrying this *Wolbachia* strain do not show the same reduction in fitness as those carrying *w*MelPop. *w*Mel has also been fixed into a naïve population in semi-field trials. Open field trials releasing *A. aegypti* infected with *w*Mel have seen persistence of the infection within the population for more than two years. This represents a good first step towards driving traits into a population however *w*Mel only impacts on the DENV-2 serotype and not the other three. It does have a refractory effect on Zika and Chikungunya.

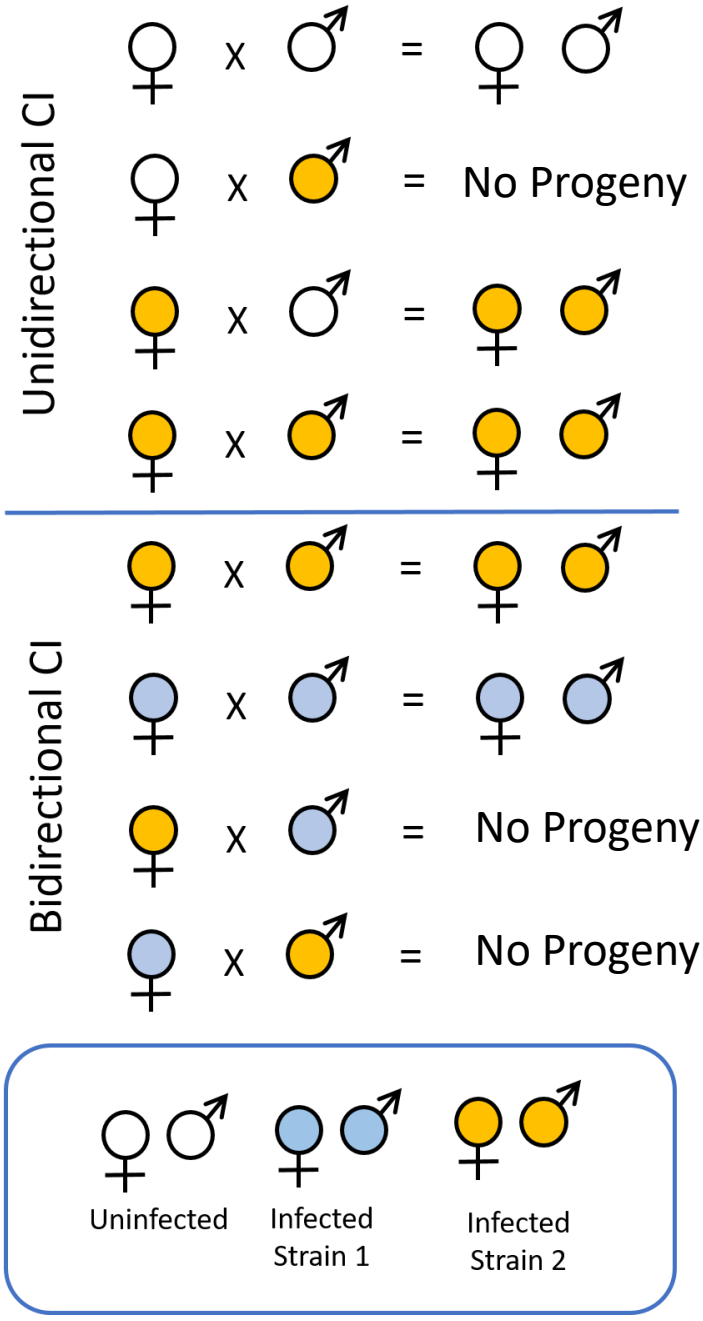


Figure 3.6

Unidirectional cytoplasmic incompatibility (CI) occurs when there is one *Wolbachia* strain in the population. When an uninfected male mates with an infected female all of the offspring carry the strain. When an infected male mates with an uninfected female there is CI and there are no viable offspring.

Bidirectional CI occurs when there are multiple *Wolbachia* strains in the population. When matings occur between individuals carrying different strains there are no viable offspring. In this case the strain that reaches the majority will continue on to fixation. [Adapted from Alphey (2014)

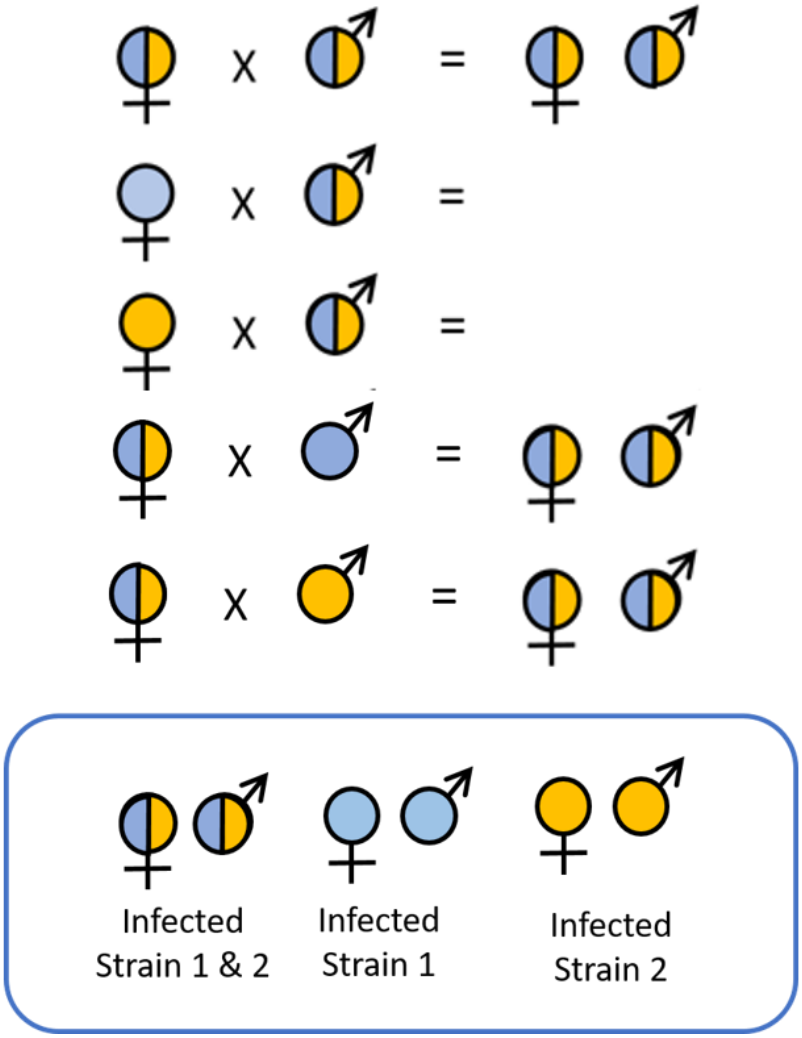


Figure 3.7

Two incompatible strains are co-infected in the same individual generating a super-infection. Super-infected females can mate successfully with males carrying either strain. Super-infected males cannot generate progeny with females carrying only one strain due to a lack of the 'rescue factor'. This means that the superinfection will reach fixation in a population that only carries one of the strains. In this way a modified strain of choice could be driven in to the population.

[Adapted from: Alphey (2014)

3.1.4.4 Self-limiting Technologies

Self-limiting technologies represent the most developed novel genetic vector control technique. The premise here is that males are released carrying a dominant lethal gene. When these males mate with wild females the lethal gene is expressed causing mortality before resulting progeny can reach adulthood. To maintain stocks and rear substantial numbers of males carrying the dominant lethal gene in the laboratory the gene must be suppressed in the lab (Black *et al.* 2011).

Currently suppression is achieved using the Tet-OFF system. The tetracycline responsive trans-activator (tTAV) is expressed downstream of a minimal promoter. It recognises a specific operator sequence (TetO). TetO is placed upstream of the lethal gene. tTAV binds to TetO and drives expression of the lethal gene resulting in mortality. If the mosquitoes are reared with tetracycline in the water, then tTAV cannot bind to TetO and the lethal gene is silenced (Figure 3.8). This enables the mosquitoes to be reared through to adulthood. Alternatively, TetO can be placed upstream of tTAV. This creates a positive feedback loop that results in over expression of tTAV and ultimately cell lethality (Phuc *et al.* 2007) (Figure 3.9).

The homozygous strain is propagated in a mass rearing facility with tetracycline added to the rearing water to suppress the dominant lethal gene. At the pupal stage males are sorted mechanically from the females based on size. These males are then released into the wild where there is not sufficient tetracycline in the environment to suppress the lethal gene (Massonnet-Bruneel *et al.* 2013). This system has proved extremely successful in field trials achieving over 90% suppression of the local population in Brazil (Alphey 2014), Malaysia (Lacroix *et al.* 2012) and the Cayman Islands (Harris *et al.* 2012).

The first-generation technology, named #513A, contains a lethal tTAV positive feedback loop that kills both males and females prior to eclosion. It also contains a red fluorescent marker to allow for the easy identification of transgenic mosquitoes from wild-type mosquitoes. #513A males are sorted mechanically as there is no genetic switch to selectively kill the females. This can have costs on fitness and does not necessarily guarantee a 100% male release. Any changes to the rearing conditions will have a subsequent effect on pupal size and reduce the effectiveness of mechanical sorting.

One way to overcome this is to generate a product strain that is female-specific eliminating contamination risk associated with mechanical sorting of pupae. These genetic sexing systems (GSS) represent the 2nd generation technology for Oxitec and are currently under development for *Aedes aegypti* and *Aedes albopictus*. #5034 is the leading strain for a GSS in *Aedes aegypti* and relies on sex specific splicing of the *doublesex* gene to limit expression to female

mosquitoes. This is combined with the tet system in such a way that only females express functional tTAV which feeds into a positive feedback loop and over-expression of tTAV, leading to death. tTAV is silenced in the lab by the addition of tetracycline to the rearing water. This would guarantee male only releases as tetracycline would not be present in the rearing water for the release generation. The females would die. The males would be released to mate with wild females and resulting female progeny would not survive to adulthood. This would skew the population and cause decline and ultimately suppression following enough releases.

Whilst this system is an attractive first step it is less appealing as a product as half of the progeny survive and so is not truly self-limiting as the males will persist in the environment carrying the genetic components to subsequent generations. Whilst they can be identified with the presence of a fluorescent marker and can be monitored using ovitraps it is more appealing to present a product that does not persist in the environment and as such is an ecologically reversible solution.

A solution to this problem is to use two parallel systems within the same insect (Figure 3.10). One to control lethality in the field and one to control sex specific lethality in the mass rearing unit. The secondsystem should ideally contain few parts, be independent of the Tet-Off system and be physiologically inert.

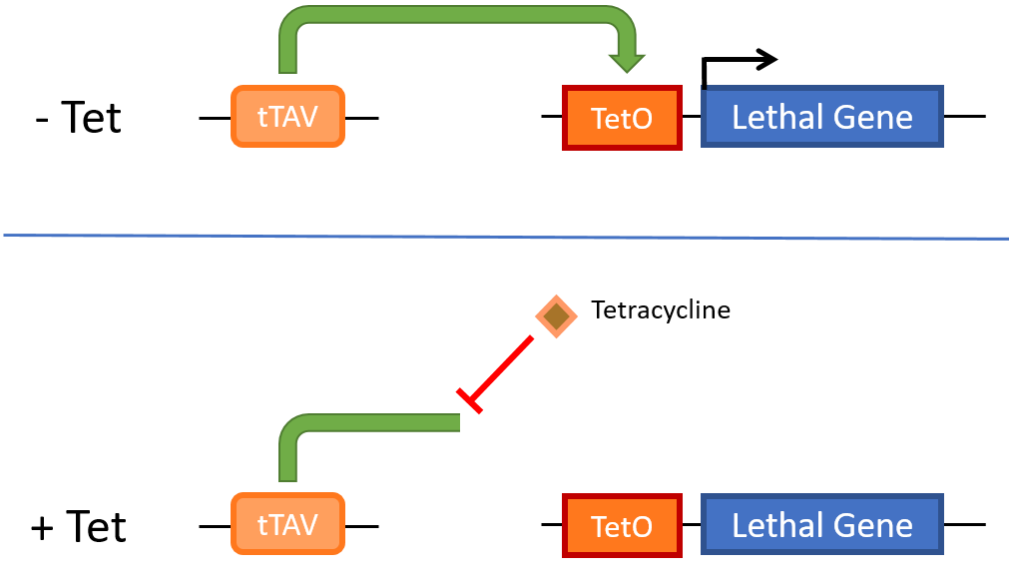


Figure 3.8

The tetracycline trans-activator (tTAV) is under the control of a specific promoter.

– Tet: tTAV is produced, this binds to the operator sequence (TetO) driving transcription of the lethal effector gene leading to death of the individual.

+ Tet tTAV is still produced however it cannot bind to TetO due to conformational changes caused by the binding of tetracycline. This silences the lethal effector gene and the individual survives.

Adapted from Gong *et al.* (2005)

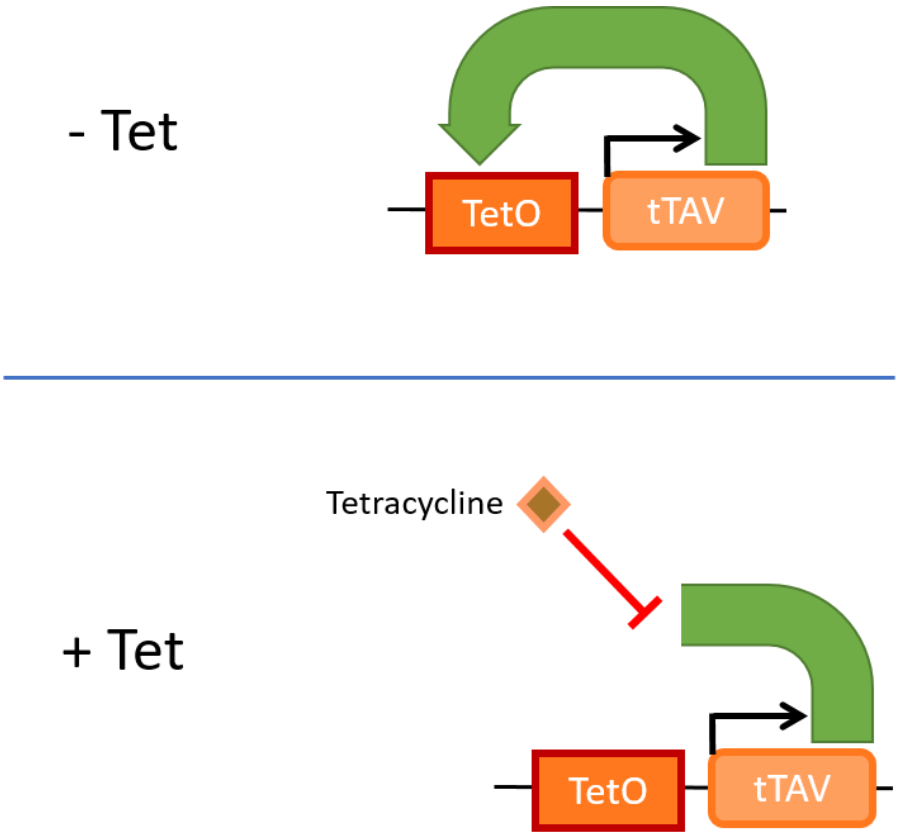


Figure 3.9

Here the lethal effector is tTAV itself which is over-expressed to toxic levels. In this configuration tTAV is serving as both the driver and the effector.

– Tet: tTAV is produced at a basal level, this binds to the operator sequence (TetO) driving transcription of tTAV. This positive feedback loop leads to cytotoxic over-expression of tTAV.

+ Tet tTAV is still produced at a basal level however it cannot bind to TetO due to conformational changes caused by the binding of tetracycline. This silences the lethal effector gene and the individual survives.

Adapted from Gong *et al.* (2005)

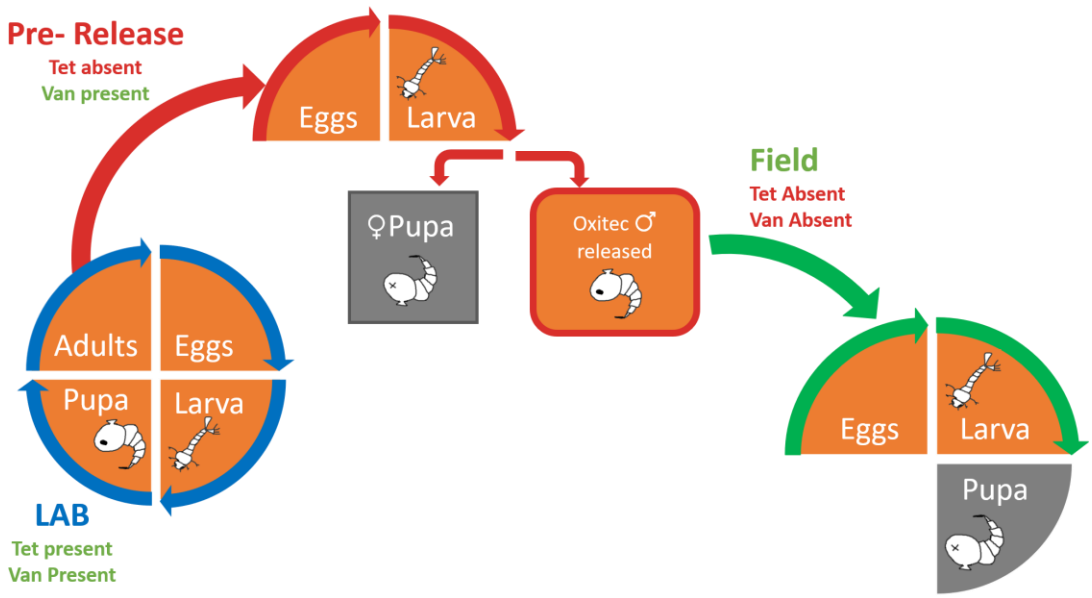


Figure 3.10

A schematic illustration of how two systems could be used in parallel to control separate parts of Oxitec's self-limiting technology. In this example all the reared and released mosquitoes carry two transgenic systems. A female-specific lethal transgene under the control of the Tet system and a universally lethal gene under the control of the vanillic acid system.

In the lab (blue arrows) mosquitoes are reared in the presence of tetracycline and vanillic acid. The two lethal transgenes are silenced, allowing for mass rearing of mosquitoes prior to release.

The release generation (red arrows) is hatched in water containing only vanillic acid. As there is no tetracycline present the female lethal gene is not supressed however the universally lethal gene is suppressed. This kills off all female pupae but allows male pupae to survive to adulthood.

Adult males are released into to field (green arrows). They mate with wild females who lay eggs in water that does not contain either vanillic acid or tetracycline and so pupae of both sexes die crashing the local population.

3.1.5 New Technology from *Caulobacter crescentus*

Caulobacter crescentus is a gram-negative bacteria characterised by an asymmetric cell division that gives rise to physiologically distinct daughter cells: a motile swarmer cell and a stationary stalked cell (Poindexter 1964). It is common to oligotrophic fresh water environments where the primary source of organic carbon is decaying plant matter (Benner *et al.* 1988). Lignin represents the second most abundant component of the vascular plant cell wall after cellulose. It has a complex chemical structure and exists as a highly cross-linked heterogeneous polymer. The process of lignin degradation is initiated by fungi (Martínez *et al.* 2005). Oxidative cleavage of lignin releases several soluble phenolic intermediates which can be metabolised by a variety of bacterial species and used as a sole carbon source. The compounds are first converted to protoatechuate by removal of the o-methyl ring. The dihydroxylated benzene ring is then broken down through subsequent reactions in the β-ketoadipate pathway; this yields succinyl CoA and acetyl CoA which can be fed into the citric acid cycle (Harwood and Parales 1996). A commonly released phenol is vanillate (4-Hydroxy-3-methoxybenzoate). This and its derivatives can serve as a sole carbon source for *C. crescentus* once it has been oxidised to protoatechuate (Thanbichler *et al.* 2007).

The initial demethylation of vanillate is carried out by two proteins, VanA and VanB. Combined they form the monooxygenase responsible for the oxidation of vanillate. They are controlled at the transcriptional level by the repressor protein, VanR. VanR binds to a specific palindromic operator sequence, VanO (ATTGGATCCAAT), upstream of the VanAB gene cluster and prevents transcription. Vanillate binds to VanR and relieves repression of the VanAB genes (Brunel and Davison 1988; Nishimura *et al.* 2006; Thanbichler *et al.* 2007). As well as vanillate, *C. crescentus* can metabolise many related compounds including vanillic acid, a common food additive (Thanbichler *et al.* 2007).

Gitzinger *et al.* (2012) engineered a synthetic switching system in mammalian cells comprising the vanillic acid responsive transcriptional repressor (VanR) and its binding site (VanO) from *C. crescentus* (Figure 3.11). The operator is placed upstream of the target gene and its expression is controlled by the presence of vanillic acid. The switch was developed in an ON and OFF configuration. The Van-ON configuration mimics the wild-type system where vanillic acid is required for gene expression. VanR binds to VanO repressing transcription, in the presence of vanillic acid VanR is released and transcription of target genes can continue. The Van-OFF configuration is achieved by converting the VanR repressor into an activator, analogous to the Tet-Off system. VanR is C-terminally fused to the VP16 trans-activator from *Herpes simplex*, forming VanTA. In the absence of vanillic acid VanTA binds to VanO and transcription of target

genes is initiated. When vanillic acid is introduced, it binds to VanTA and transcription is silenced. The initial study demonstrated that both configurations were dose dependent, highly specific and fast acting in mammalian cells. This system has been integrated into a higher order control network alongside the Tet-O system (Folcher *et al.* 2013). A recent study integrated the TetO and Vanillic acid system in *Trypanosoma brucei*.

The VanTA system would be ideal for use in insect transgene control. It has a minimal number of components and is controlled by a commonly used and readily available food additive (Sinha *et al.* 2008). This system is of particular interest for furthering work at Oxitec as it has already been integrated alongside the molecular switch system they already employ, the Tet-O system. It is also highly unlikely that vanillic acid would be available in the wild mosquito diet at sufficient quantities to affect transgene expression.

3.1.6 Towards a Vanillic Acid Switch in *Aedes aegypti*

The Van-Off system could be used by Oxitec Ltd. alongside the Tet-Off system to control separate essential mechanisms for the self-limiting approach to insect population control. The Van-Off system could be combined with female-specific lethality from 2nd generation lines. Vanillic acid would be required to maintain the population for research and mass rearing, however if vanillic acid is not present in the release generation then the females would die leaving the males.

In mosquitoes carrying a Van-OFF GSS and a Tet-OFF lethal response system both vanillic acid and tetracycline are required to maintain stock populations. The release generation would be reared only in tetracycline; this would result in female death as there is no vanillic acid to supress the GSS however the males would survive. The males would then be released to mate with wild females and all progeny would die as there is no tetracycline in the environment.

This chapter presents the first steps towards a functional Van-Off switch in the mosquito vector *Aedes aegypti.*

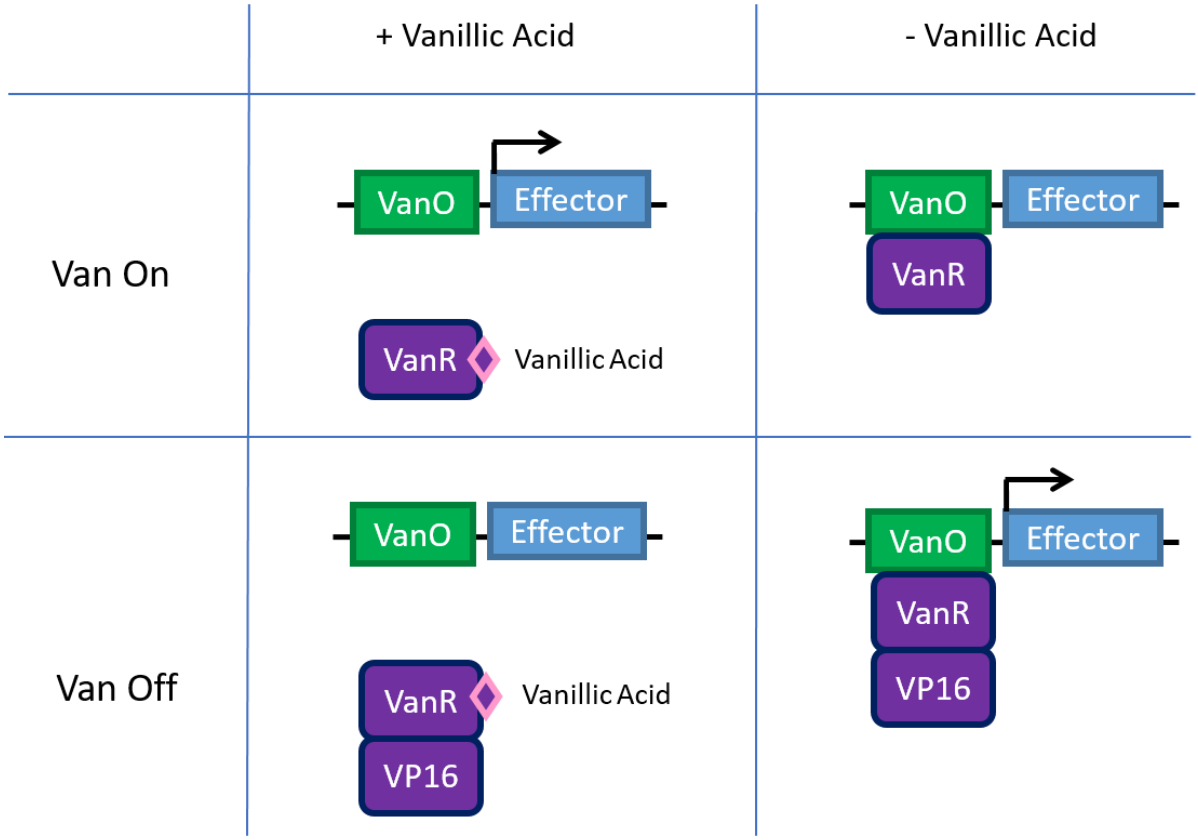


Figure 3.11

The vanillic acid responsive system created by Gitzinger *et al.* (2012).

Van On: When vanillic acid is present it binds to the repressor (VanR) releasing suppression of the effector. When vanillic acid is absent from the system VanR recognises the operator (VanO) and binds to it silencing the effector gene.

Van Off: VanR is c-terminally fused the trans-activator from *Herpes simplex*. When vanillic acid is present it binds to VanR and silences transcription of the effector. When vanillic acid is absent VanR-VP16 is free to bind to VanO and VP16 recruits transcriptional machinery to drive expression of the effector.

3.2 Methodology

3.2.1 Mosquito Rearing

Mosquitoes were reared at 27^{o}C (±1^{o}C) and 70% relative humidity (±10%) throughout their lifespan. Larvae were reared in plastic trays filled with deionised water. Larvae were fed on crushed dry fish food (TetraMin flake food from Tetra Werke, Germany). This was done by eye for the purposes of stock maintenance however during controlled experiments food was weighed out following an optimum feeding regime (Table 3.1). Larvae were ideally reared at 1 larva/ml with 1000 larvae per tray however unless stated this was an approximation. If trays were too densely populated excess larvae were removed. Pupae were picked using a plastic pipette with a small portion of the tip removed and sexed under a binocular microscope. If required pupae were also phenotyped under a fluorescent microscope.

Male and female pupae were placed in a 100 ml weighboat, filled with deionised water, inside an insect rearing cage (10 cm^{3}) and left to eclose. Ideally there was a 2:1 ratio of males to females within the cage to allow for limited competition. Adults were fed on 10% sucrose solution containing streptomycin 14 μg/ml and penicillin 14 U/ml. The antibiotics helped to preserve the life of the sugar feeders by preventing the growth of mould and bacteria. Females were fed on horse blood using metal plates covered in parafilm, these were warmed using bean bags. Plates were left on for several hours and cages were provided with blood plates every few days. Eggs were collected on seed paper, a rough brown paper used in horticulture. The paper was placed in a 100 ml weighboat that was half-filled with deionised water. The papers were removed after two days, to prevent hatching. Excess water was poured off and the papers were left to dry on a shelf in the insectary. Once dried the paper can be stored in the insectary for up to three months. In order to hatch the eggs several papers were placed in a plastic 500 ml deli pot. The papers were covered in milli-Q water and hatched under a vacuum for at least two hours.

3.3.2 Plasmid Construction

Plasmids were designed and synthesised by the Oxitec molecular team. Full maps of all the constructs are included in Appendix 2. In total four constructs were synthesised by the Oxitec molecular team for this work #5112 (HexVanTA), #5120 (VanODsRed2), #5161 (HexVanTA2.0) and #7178 (HexVanTA2.1). The VanTA present in #5178 was optimised using GeneOptimizer® from Life Technologies. Prior to injection maxi-preps were performed to amplify plasmid DNA (EndoFree Plasmid Maxi Kit) and then sequenced by Sarah Scaife to check for mutations in the plasmid sequence.

3.2.3 Transformation of *A. aegypti* Eggs

Preblastoderm *A. aegypti* [Latin strain] G0 embryos were collected and prepared for microinjection. A densely filled cage of bloodfed females was placed in the dark for an hour to allow females a chance to lay eggs. Unlike normal egg collection a weighboat is filled with cotton wool and wetted, a white circular filter pad was placed on top as flat as possible. After an hour the egg paper was removed and checked for eggs. Using tweezers eggs were transferred to a slide covered in double sided sticky tape and left to begin hardening. Embryos were injected with a mixture of construct DNA (500 ng/µl) and *piggyback* mRNA (300 ng/µl) at the apical end. Slides of injected eggs were placed vertically in a pot of water to allow excess oil to drain off. Slides were then stored in a sealed humid box for three days. At the end of the three days the rack of slides was placed in a deli pot of deionised water and placed under a vacuum to hatch. HexVanTA (#5112) and VanODsRed2 (#5120) were injected by Pam Gray (Oxitec). HexVanTA2.0 and HexVanTA2.1 were injected by Emily M^{c}Gann and Ilona Flis (Oxitec).

Surviving G0 males were crossed individually to 5 -7 females from the host strain. After being left for two days to mate pools were established containing 3 G0 males and the wild type females. All surviving G0 females were pooled together and crossed to background strain males. Resulting G1s were screened for the marker gene at L3 and pupal stages using a Leica M80 microscope and a Prior Lumen 200 fluorescence illumination system. Transgenic lines were then established from positive pools. Where possible three single male crosses were performed. Additional transformants were pooled. G2s from the single male crosses were screened, sexed and counted at the pupal stage to determine whether individual lines had multiple insertions and / or were sex-linked. #5120 and #5112 lines were established by Pam Gray and Sean Marren. #5161 and #5178 lines were established by Emily M^{c}Gann.

3.2.4 Vanillic Acid Assay

Insects carrying HexVanTA (#5112, #5178 and #5161) were crossed to individuals carrying VanODsRed2 (#5120). The eggs were hatched under a vacuum and L1s from each cross were separated into six cohorts of 200 larvae. Larvae were counted individually into deli pots using a glass pipette. 200 mL of milli-q water was added to three of the pots, to the other three pots 200 mL of water containing 60 µg / mL vanillic acid (Sigma Aldrich) was added. In total 600 larvae were reared in control conditions and 600 in water containing vanillic acid. If the hatch rate was poor then as many L1 larvae were counted as possible and split equally between the six pots. Larvae were fed according to the standard feeding schedule (Table 3.1). Pupae were picked daily and then sexed and screened for the three fluorescent markers (red eyes, blue bodies and red bodies). This continued until 3% or less of the starting cohort remained.

3.2.5 Molecular Analysis:

Three L3 larvae per line examined were used for RNA extractions. RNA was extracted using the Norgen purification kit (Cat no. #17200), following the manufacturer's instructions except that between the two 400 µl washes RNA was treated with Norgen RNase-free DNase I kit (Cat no. #25710). cDNA was synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Transcripts were analysed with primer pairs designed by Tarig Dafalla and Sarah Scaife of the Oxitec molecular team (Table 3.2). cDNA was then amplified by RT-PCR (PCRBio Taq Polymerase) using the parameters listed in Table 3.3. Following the completion of the programme products were run on agarose gels (1.2%, 120 V, µl of product and 5µl of bromophenol blue) alongside 5 µl of SmartLadder (Eurogentec MW-1700-10) and relevant controls. Gels were then visualised under UV and photographed.

3.2.6 Statistical Analysis

All statistical analysis was conducted using RStudio running R version 3.3.2. To determine whether there were multiple insertion events or sex-linkage in the new transgenic lines a Fisher's exact test was performed on a two by two contingency table composed of the categories for sex (male and female) and phenotype (transgenic or wild-type like). Following the initial vanillic acid assay an exact binomial test was used (two-tailed) to ascertain if the expected Mendelian ratios were seen in the progeny when reared in water and when reared in vanillic acid. A one-tailed exact binomial test was used to determine if either HexVanTA2.0 and HexVanTA2.1 had a detrimental effect on survival to pupation compared to a wild type control. A two-sample t-tes used to determine whether rearing condition (in pure water or in water containing vanillic acid) had a significant impact on the mean number of individuals surviving to pupation. Finally, a Fisher's exact test was used to determine whether there was a significant difference between the number of individuals expressing the red bodies phenotype between the two rearing conditions.

Table 3.1

Feeding regimen for larvae during on/off vanillic acid assay: the amount of Tetramin (g) fed to cohorts of 200 larvae.

Day	Tetramin Added (g)
1	0.012
2	-
3	0.016
4	0.032
5	0.064
6	0.064
7	0.064
8	0.064
9	0.032
10+	As required

Table 3.2

Primer pairs designed by Sarah Scaife (VP16) and Tarig Dafalla (Muscle Actin 4)

	Forward	Reverse	Product Size
VP16	5'-*gtttcgtgcggagatgctga*-3'	5'-*ggtaaacatctgctcaaactcgaagtc*-3'	502 bp
Muscle Actin 4	5'-*cccaggaaggatggctggaagag*-3'	5'-*ggcattcattctagttccgaaaccg*-3'	881 bp

Table 3.3

RT-PCR parameters

1	94°C	55 seconds
2	95°C	55 seconds
3	55°C	30 seconds
4	72°C	30 seconds
5	Repeat from step 2 for 39 cycles	
6	72°C	120 seconds

3.3 Results

3.3.1 Vanillic Acid System in *Aedes aegypti*

To investigate the potential for a vanillic acid switch in *A. aegypti* a Van-Off test system was devised. The test system comprises a bipartite design in which the trans-activator (VanTA: VanR fused c-terminally to VP16) and the operator (VanO) were present on separate constructs. This ensures that each component can be checked individually as well as in combination. In the test system, vanillic acid is used to control the expression of a reporter gene in a manner analogous to the Tet-Off system. (Figure 3.12). In the Tet-Off system tetracycline is required to silence transcription of the target gene, in the Van-Off system vanillic acid is responsible for silencing target gene expression.

The driver construct, HexVanTA, contains the transcription unit for the vanillic acid responsive trans-activator driven by the *A. aegypti Hexamerin* promoter. *Hexamerin* is normally expressed during the late larval and pupal stages in small clusters of fat cells throughout the body (Zakharkin *et al.* 1997; Totten *et al.* 2013). This promoter has also been used successfully by Oxitec to drive ectopic expression in dominant lethal constructs. The construct carries the *Hexamerin* promoter sequence follower by its 5' UTR, then the VanR-VP16 sequence with the *Drosophila fs(1)k10* 3'UTR. The responder construct, VanODsRed2, carries the DsRed2 transcription unit including the 3' UTR from *AeaHsp83*, downstream of four tandemly arranged VanO sequences and the *hsp70* minimal promoter and the *hsp70* 5' UTR. The lines are typically maintained in a heterozygous state, with selection every generation for transgenic progeny based on the transformation marker. When mosquitoes carrying the individual constructs are crossed to each other a quarter of their progeny will carry both constructs. The VanTA should be expressed throughout the body (particularly in the fat cells) and this should bind VanO and drive expression of DsRed2, resulting in red fluorescence throughout the body when viewed under fluorescent light. When the larvae are reared in the presence of vanillic acid this should bind to VanTA and prevent it from binding to VanO. This would then result in silencing of expression of DsRed2, and no fluorescence (Figure 3.13).

Each construct also contained a separate transformation marker to identify transgenic animals carrying either the VanTA, VanODsRed2 or both. HexVanTA is marked by a fluorescent red eyes phenotype where DsRed2 is expressed under the 3xP3 promoter which promotes expression exclusively in the eyes. The VanODsRed2 construct is identified by a blue fluorescent bodies phenotype, the fluorescent marker AmCyan is driven by the ubiquitously expressed Hr5IE1 promoter. If the system works as designed, individuals carrying both constructs should express fluorescent red eyes, fluorescent blue bodies and fluorescent red bodies when reared without vanillic acid, whereas those reared in vanillic acid should only express the two marker phenotypes: fluorescent red eyes and fluorescent blue bodies (Figure 3.14).

3.3.2 Initial Constructs and Vanillic Acid Assay

The first two constructs #5112 (HexVanTA) and #5120 (VanODsRed2) were injected by Pam Gray and Sean Marren (Oxitec) and lines were established, five for HexVanTA and three for VanODsRed2. Initially ten crosses were established between the HexVanTA and VanODsRed2 lines generating G_3 offspring containing both constructs. Offspring from these crosses were hatched and reared in a vanillic acid assay. L1's were hand-picked and reared in pots containing 60 µg / ml vanillic acid at a density of 1 larva / ml. A control was run alongside with L1's reared in pure water at the same density. Individuals were picked and sexed under a microscope at pupation and then discarded. Expression of fluorescent red bodies was not detected in any of the offspring carrying both the VanODsRed2 and VanTA constructs irrespective of rearing conditions (Figure 3.15). The majority of crosses did not yield the expected mendelian ratio of phenotypes (Table 3.4) however all expected phenotypes were seen for each cross. This indicates that there is insufficient expression of the VanO-DsRed2 construct to generate detectable fluorescence in any conditions.

3.3.3 Transcription of the VanR-VP16 Cassette

The initial tests indicated that the system is not working as predicted. To determine which component(s) are non-functional RT-PCR analysis was carried out on several of the HexVanTA lines to determine whether there was expression of the VP16 cassette in larvae. All tested lines showed expression of the VanR-VP16 transcript (Figure 3.16) in at least one of the tested samples. This indicates that this component of the system is being transcribed.

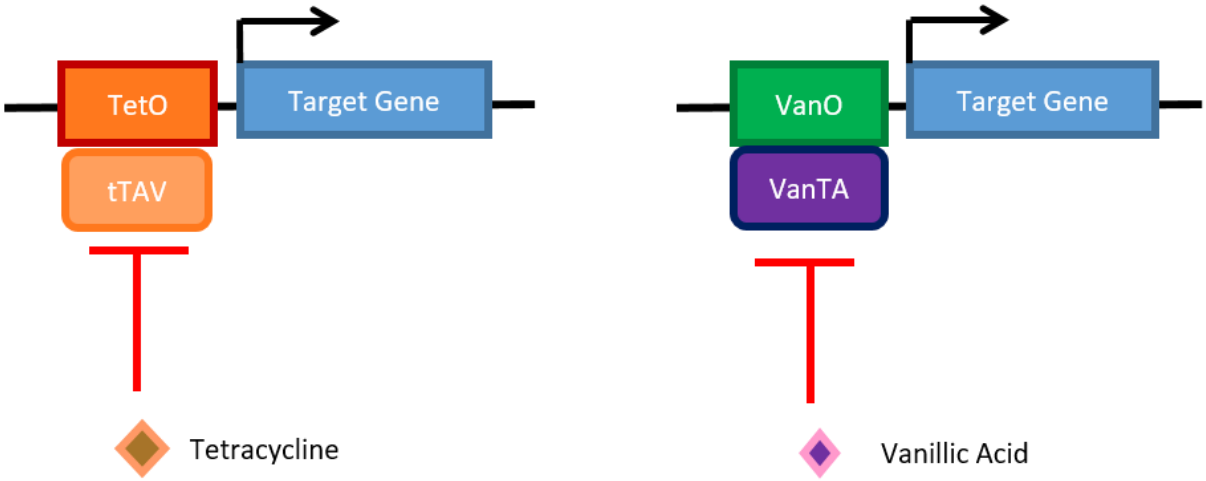


Figure 3.12

The proposed vanillic acid system shares many analogous features to the Tet-Off system currently employed by Oxitec. The transactivator (tTAV / VanTA) binds to its specific operator (TetO / VanO upstream of the Target Gene and drives transcription. In the presence of the effector molecule (tetracycline / vanillic acid) transcription is silenced as the trans-activator can no longer bind to its specific operator.

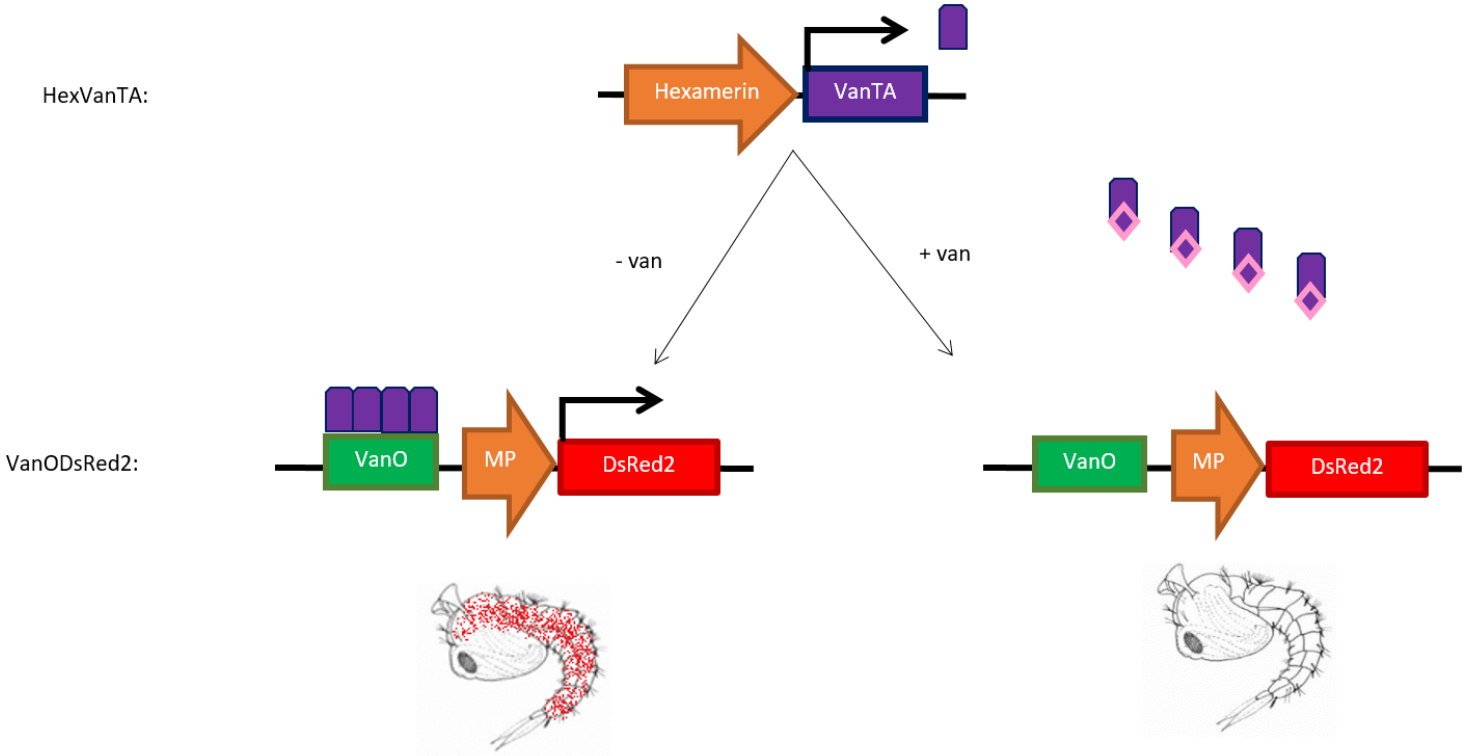


Figure 3.13
Schematic diagram detailing a bipartite vanillic-off system (Van-off). HexVanTA contains the vanillic acid responsive trans-activator, VanTA, downstream of the Hexamerin promoter. This drives expression of VanTA across the body of the pupa. When reared without vanillic acid dissolved into the rearing water (-van) VanTA is free to bind to VanO, there are four repeats of the operator present on the VanODsRed2 construct. This drives expression of the fluorescent protein DsRed2. This is seen as red fluorescence across the pupal body. When reared with vanillic acid dissolved in the rearing water (+van) VanTA cannot bind to VanO due to the binding of vanillic acid. The transcription of DsRed2 is prevented and there is no fluorescence shown across the body.

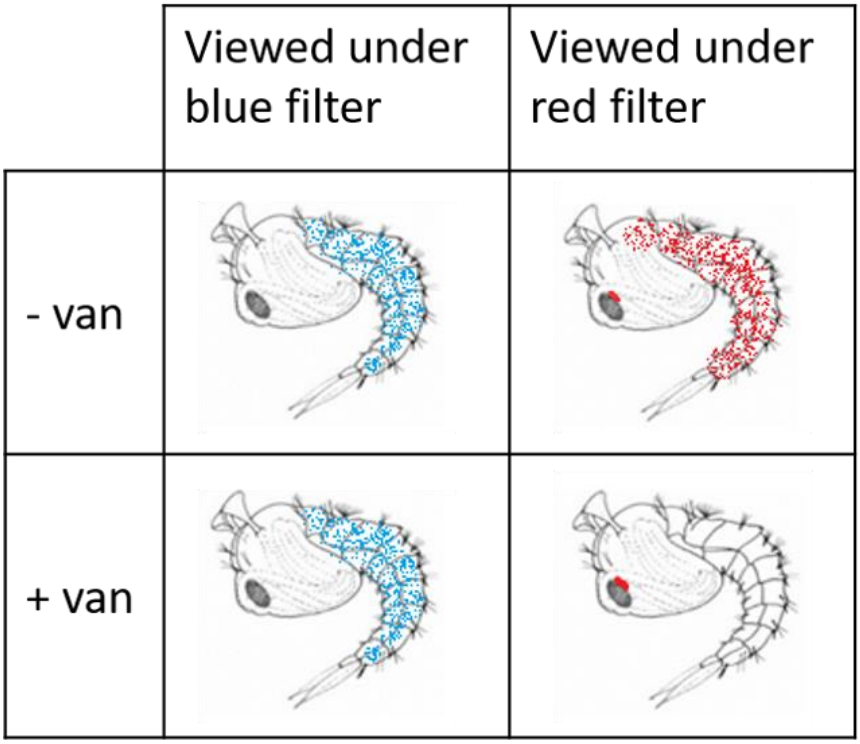


Figure 3.14

Each construct can be identified by the presence of a marker gene. HexVanTA is identified by red eyes (3xP3 – DsRed2) and VanODsRed2 is marked by blue bodies (Hr5iE1-AmCyan). Individuals carrying both constructs should express both of these markers as well as VanTA driven red body fluorescence (Hexamerin-DsRed2) when raised without vanillic acid (-van). When reared in the presence of vanillic acid (+van) then only the two fluorescent marker phenotypes will be visible: red eyes and blue bodies.

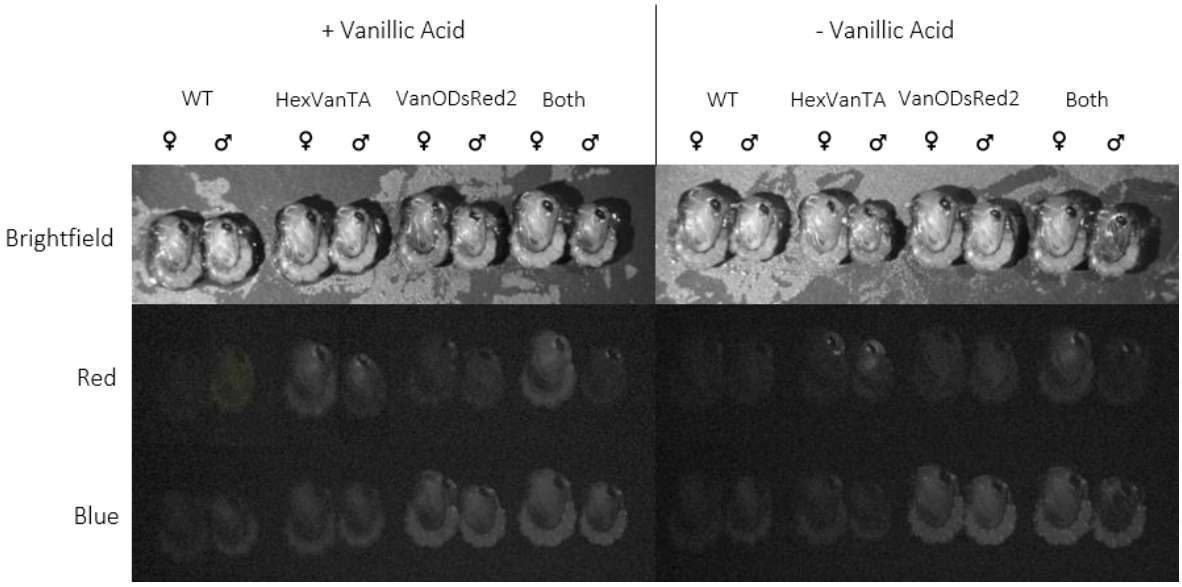


Figure 3.15

Results of on/off vanillic acid assay. Progeny from cross 1C are shown (HexVanTA-1 crossed to VanODsRed2-C) under bright light, red filter and blue filter. The four expected marker phenotypes are represented. Whilst the transformation markers are clearly expressed in both sexes, an example of red eyes is indicated by the white arrows. There is no expression of red bodies in pupae carrying both constructs, irrespective of rearing conditions.

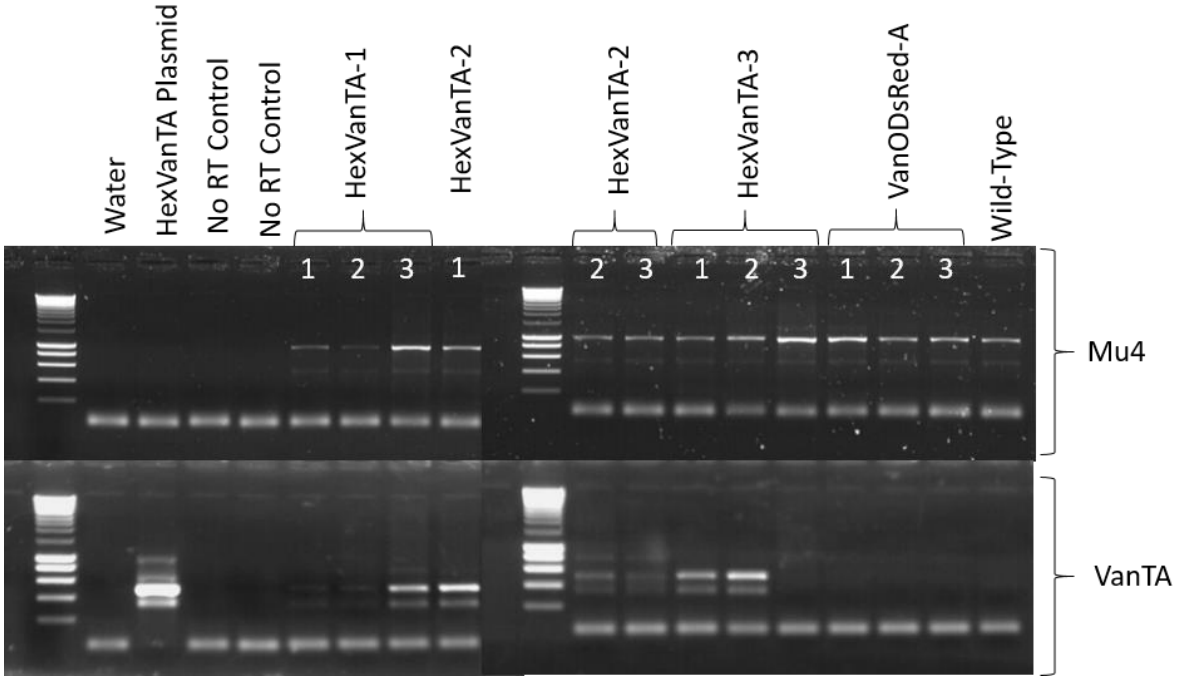


Figure 3.16

Gel of PCR amplifying VanTA sequence from the HexVanTA construct. Bands for the control gene muscle actin 4 (Mu4) are at the correct apparent molecular weight on the gel. VanTA is present in all three HexVanTA lines and not present in VanODsRed-A.

Table 3.4

Results of a G-Test to determine whether the ratio of phenotypes adhered to the expected 1:1:1:1 ratio. Significant results are highlighted in bold.

Cross	Reared in Vanillic Acid		Reared in Water	
	P-Value	G-Value	P-Value	G-Value
1A	**0.0143**	10.57	0.0969	6.324
1C	**<0.001**	134.50	**<0.001**	123.290
2A	**<0.001**	218.65	**<.0001**	195.600
2B	**<0.001**	136.40	**<0.001**	98.133
3B	0.3555	3.24	0.6851	1.488
3C	**<0.001**	51.50	0.772	1.121
4A	**<0.001**	119.68	**<0.001**	110.380
4B	**0.0086**	11.67	**0.0011**	16.153
5A	0.1143	5.94	0.9035	0.569
5B	**<0.001**	46.66	**<0.001**	23.145

3.3.4 Correction of HexVanTA

Following the detection of the transcript, the original plasmid design sequence was scrutinised. An in frame stop codon was identified between the VanR and VP16 sequences, a restriction enzyme site was also identified that was causing a frame shift in the VP16 portion of the fusion protein (Figure 3.17). A literature search was conducted to identify the exact VanTA sequence used by Gitzinger *et al.* (2012). The functional VanTA used by Gitzinger *et al.* contained the VanR sequence directly from the *C. crescentus* genome followed by a single serine, the terminal amino acid from the TetR-VP16 fusion protein, then the VP16 sequences from *Herpes simplex* (Gossen and Bujard 1992; Fussenegger *et al.* 1997; Gitzinger *et al.* 2012) HexVanTA2.0 was generated with a new VanTA sequence, seven bases were deleted that removed the stop codon and placed the VP16 portion back in frame (Figure 3.17).

3.3.5 Injection of the HexVanTA2.0 and HexVanTA2.1 Constructs

The corrected VanTA sequence was codon optimised for use in *A. aegypti* by Tarig Dafalla (HexVanTA2.0); an alternate optimisation was generated with GeneOptimizer® (Life Techonologies) using *D. melanogaster* as a template organism (HexVanTA2.1). The two synthesised constructs were injected separately into pre-blastoderm embryos, in conjunction with the piggybac helper plasmid to facilitate transformation. Overall survival of injected embryos to adulthood for HexVanTA2.0 was 3.9% and for HexVanTA2.1 was 3.3% (Table 3.5).

3.3.6 Establishment of Lines for HexVanTA2.0

Following injections and crosses of G0 mosquitos in pools, surviving G1s were screened at the L3/L4 and pupal stages of development to check for presence of marker expression. 6 out of the 19 pools contained transgenic animals. This led to the generation of 17 lines, of which at least 6 were independent. G2s were screened and phenotypes for the lines were compared. All lines expressed the marker in a similar distribution and with similar intensity. Following this each line was tested to determine whether it contained a single transgene insertion, or if there were multiple insertions. Single transgenic males were crossed to wild type females and offspring from were sexed and screened for the marker phenotype. A Fisher's exact test was performed, values closer to 1 indicate a ratio of 1:1:1:1 between males and females expressing the marker and not expressing the marker. Lines with the highest P-values were carried forward (Table 3.6). Lines 13B, 16C, 18C and 19C failed generate viable progeny in this test. Lines 6A, 6B and 6C appear to be sex linked, since only male transgenics were recovered, and were discounted. Lines 4C, 13A, 16A, 18B and 19B were kept.

3.3.7 Establishment of Lines for HexVanTA2.1

11 pools of G0s were established, 9 from male injection survivors and 2 from female injection survivors. 5 of these pools gave rise to G1 transformants and 14 lines were generated from these pools. Following screening of G2s, line 2C was split into a bright and a dull phenotype (2CB and 2CD respectively). A fisher's exact test was used to determine which lines should be carried forward. Following single male crosses, offspring were scored based on sex and phenotype as above. Lines 5B and 7A failed to generate viable progeny. Lines 7B and 7C were determined to be sex linked as no females expressed the fluorescent red eyes phenotype. Lines 1A, 1C, 2CD, 3A and 5C were selected for further crosses (Table 3.6).

3..3.8 Crosses of HexVanTA2.0 and HexVanTA2.1 to VanODsRed2

Table 3.7 details which HexVanTA lines were crossed to the existing VanODsRed2 lines and tested in a vanillic acid assay. The process for pairing lines together was random. Each HexVanTA2.0 line to crossed to at least one VanODsRed2 line. Subsequent crosses (orange) were performed to determine which part of the system was performing better in terms of expression of the fluorescent red bodies phenotype. Two of the HexVanTA2.1 lines were crossed to VanODsRed2-C.

A) cgt gcg gac tga ggg ccc cgc gta cag ccg
R A D *

B) cgt gcg gac tcc gcg tac agc cgc gcg cgt
R A D **S A Y S R A R**

Figure 3.17
The sequence for the joining region between VanR and VP16 with predicted translation underneath for A) HexVanTA and B) deduced sequence from literature search. HexVanTA contains an in-frame stop codon (*) and a frame-shift. The red bases were deleted to remove the stop codon and place VP16 in-frame. The literature search revealed that the terminal amino acid from TetR-VP16 (Ser) was included for generation of subsequent transactivator proteins and so was included in the corrected sequence. Bold letters represent the beginning of VP16 including the terminal serine from TetR.

Table 3.5
Injection record for the two new HexVanTA constructs.

Construct Name	**HexVanTA2.0**	**HexVanTA2.1**
Construct No.	OX5161	OX5178
No. eggs injected	2288	1736
No. survive to pupation	104 (4.5%)	58 (3.3%)
No. survive to eclosion	90 (3.9%)	51 (2.9%)

Transgenic Pool No.	Lines Established from Each Pool		
HexVanTA2.0			
4	4A p=0.246	4B p=0.281	**4C** p=0.780
6	6A P=0.009	6B p<0.001	6C p<0.001
13	**13A** P=0.836	13B *	
16	**16A** p=0.491	16B p=0.343	16C *
18	**18B** 0.367	18C *	18D p<0.001
19	19A 0.312	**19B** 0.383	19C *
HexVanTA2.1			
1	**1A** P=0.314	1B p<0.001	**1C** p<=0.260
2	2B <0.001	**2CB** P=1.00	2CD P<0.001
3	**3A** p=0.832	3B P=0.377	3C P=0.759
5	5A 0.098	5B *	**5C** 0.110
7	7A *	7B P<0.001	7C P<0.001

Table 3.6

Following a single male cross to latin wild-type (lwt) females the number of transgenic males and females were scored. In order to determine whether there was a deviation from the expected 1:1:1:1 ratio, which would indicate sex-linkage or multiple insertion event, these numbers were entered into a Fisher's exact test. Lines in bold were carried forward for use in on/off vanillic acid assays. All other lines were terminated.

P value of the Fisher's exact test is shown. * = hatch failure of egg papers resulting from single male cross.

Table 3.7
Overview of the line names for the HexVanTA2.0 and HexVanTA2.1 constructs detailing which VanODsRed2 lines they were crossed to for on/off vanillic acid assays. The first combination of crosses are the Green boxes any subsequent crosses are highlighted in orange.

	Line No.	VanODsRed2 - A	VanODsRed2 - B	VanODsRed2 - C
HexVanTA2.0	4C			
	13A			
	16A			
	18B			
	19B			
HexVanTA2.1	1A			
	1C			
	2CD			
	3A			
	5C			

3.9 Effect on Presence of Vanillic Acid on Survival to Pupation

Alongside the transgenic lines lwt mosquitoes were also reared in water and vanillic acid to serve as a control. The survival to pupation of lwt reared in water was 61.33%. This served as a benchmark for comparison for the transgenic lines. The presence of transgenes significantly lowered the survival of individuals resulting from crosses HexVanTA2.0-18A vs VanODsRed2-B (binomial test 1-tailed: observed = 47.17, expected = 61.33, $p < 0.001$) and HexVanTA2.0-19A vs VanODsRed2-B (binomial test 1-tailed: observed = 34.67, expected = 61.33, $p < 0.001$) when reared in water. The other crosses did not show a significant reduction in the number of individuals surviving to pupation (Figure 3.18) when reared in water.

The presence of vanillic acid (60 µg/mL) in the rearing water did not significantly reduce the survival to pupation in the majority of lines (Figure 3.19). Survival was however significantly reduced in lines 4C-C, 5C-C and 3A-C.

3.3.10 HexVanTA2.0 and HexVanTA2.1 Vanillic Acid Assays

Initially five HexVanTA2.0 – VanODsRed crosses were tested in the presence and absence of vanillic acid to determine if the VanOff system is functional (Table 3.7 green cells). Progeny carrying both constructs should all express the fluorescent red bodies phenotype when reared in water. When reared with vanillic acid then none of the pupae carrying both constructs should present with fluorescent red bodies.

All five crosses showed expression of the fluorescent red bodies phenotype illustrating that VanTA is binding VanO and initiating transcription of DsRed2 (Figure 3.20 and Figure 3.21). None of the crosses showed a significant reduction in percentage of individuals expressing fluorescent red bodies between the two treatments (Figure 3.22). Fluorescent red bodies are not seen in individuals that contain only one of the two transgenes. Together this indicates that the system is efficiently activated, but is not repressible using 60 µg / ml vanillic acid.

The expression of DsRed2 in the body was most apparent in the tail of the pupae on the ventral side. There was a visible difference in expression of Hexamerin-VanTA driven DsRed2 between sexes. Expression was much stronger in females than in males. In some crosses expression of DsRed2 in males could not be seen under the microscope (Figure 3.20). All males from HexVanTA2.0 16A – VanODsRed2C expressed fluorescent red bodies visibly in the tail (Figure 3.23) although expression was noticeably reduced when visually compared to their sibling females.

16A was the only HexVanTA2.0 line initially crossed to VanODsRed2-C. To determine which part of the system was responsible for the successful turn-on rate, HexVanTA2.0-16A was crossed to VanODsRed2-B and HexVanTA2.0-4C was crossed to VanODsRed2-C (Table 3.3 orange cells). The proportion of individuals expressing fluorescent red bodies was 100% for progeny resulting from cross HexVanTA2.0 4C – VanODsRed C, this is up from around 50% of progeny expressing fluorescent red bodies in cross HexVanTA2.0 4C – VanODsRed C (Figure 3.22). Following this assay, line VanODsRed2-C was carried forward as it seemed to help abate suspected differences in sex specific expression of fluorescent red bodies

Two HexVanTA2.1 insertion lines were tested; both were successfully turned on but, as with the HexVanTA2.0 lines, there was no evidence of vanillic acid affecting the visibility of the fluorescent red bodies phenotype. The system is therefore also not switching off with this codon-optimised VanTA version (Figure 3.22). The fluorescent red bodies phenotype was visible in both sexes although, as before, females appeared brighter than males.

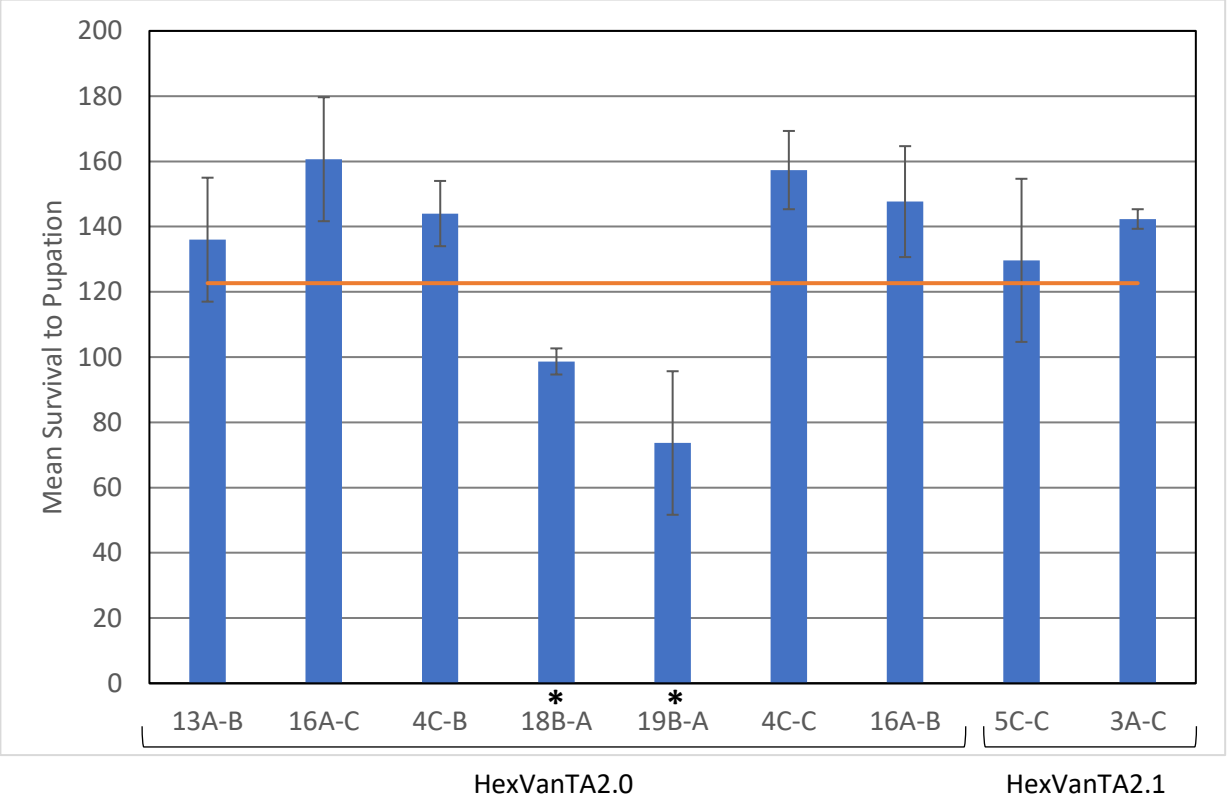


Figure 3.18

The mean number of individuals that survived through to pupation when reared in pure water. Initially 600 L1 larvae were counted into three rearing pots of 200 each. The orange line is the average latin wild-type averagewhich served as a control. Survival to pupation was significantly lower than that seen in LWT for lines 18B-A (binomial test 1-tailed: observed = 47.17, expected = 61.33, $p < 0.001$) and 19B-A (binomial test 1-tailed: observed = 34.67, expected = 61.33, $p < 0.001$).

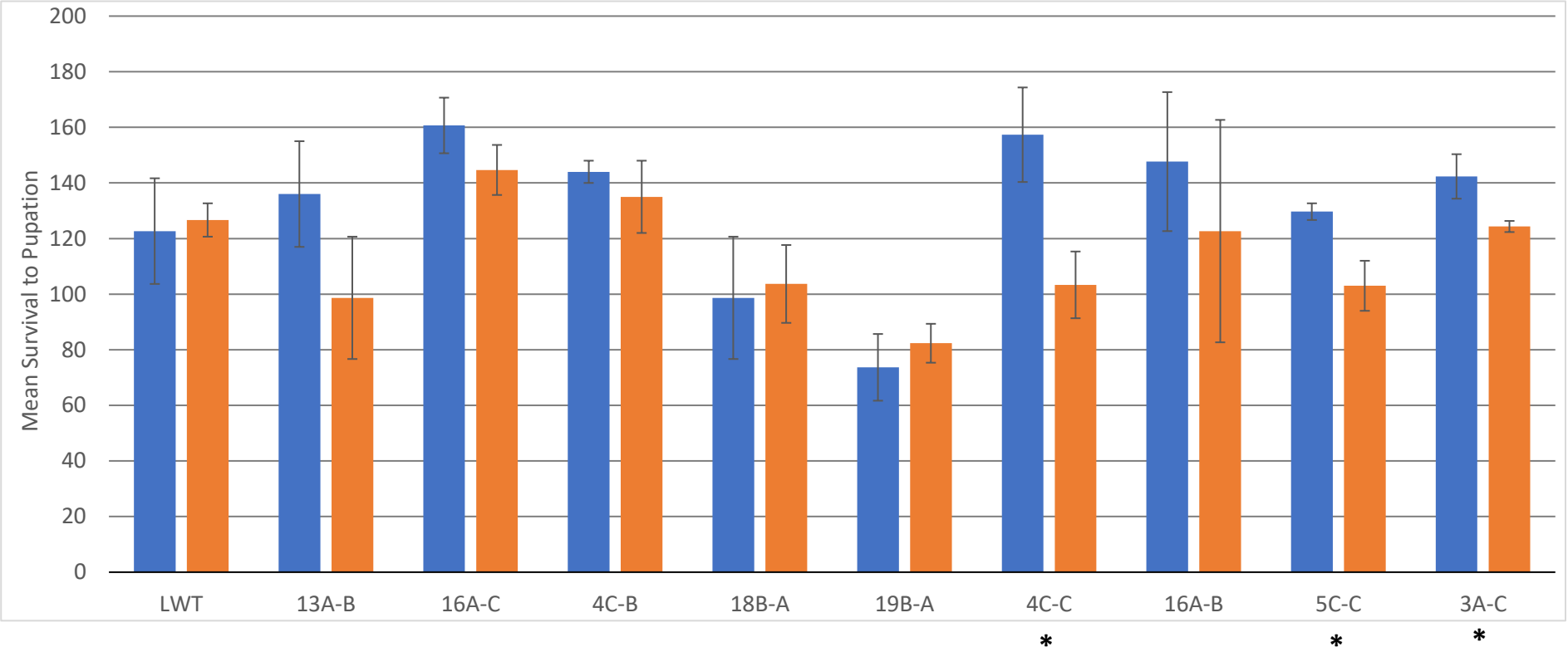


Figure 3.19

The mean number of individuals surviving to pupation when reared in pure water (blue bars) and in water containing 60 µg/mL vanillic acid (orange bars), n=3. Results are shown for a Latin wild-type control (LWT) and nine transgenic crosses (HexVanTA line number – VanODsRed2 line number). Mean survival to pupation was compared using a Two-sample t-test. Rearing in vanillic acid did not significantly lower survival in the majority of crosses however it did significantly lower survival to pupation in lines 4C-C ($t = 4.627$, d.f. = 4, $P = 0.010$), 5C-C ($t = 4.781$, d.f. = 4, $P = 0.009$) and 3A-C ($t = 4.036$, d.f. = 4, $p = 0.016$).

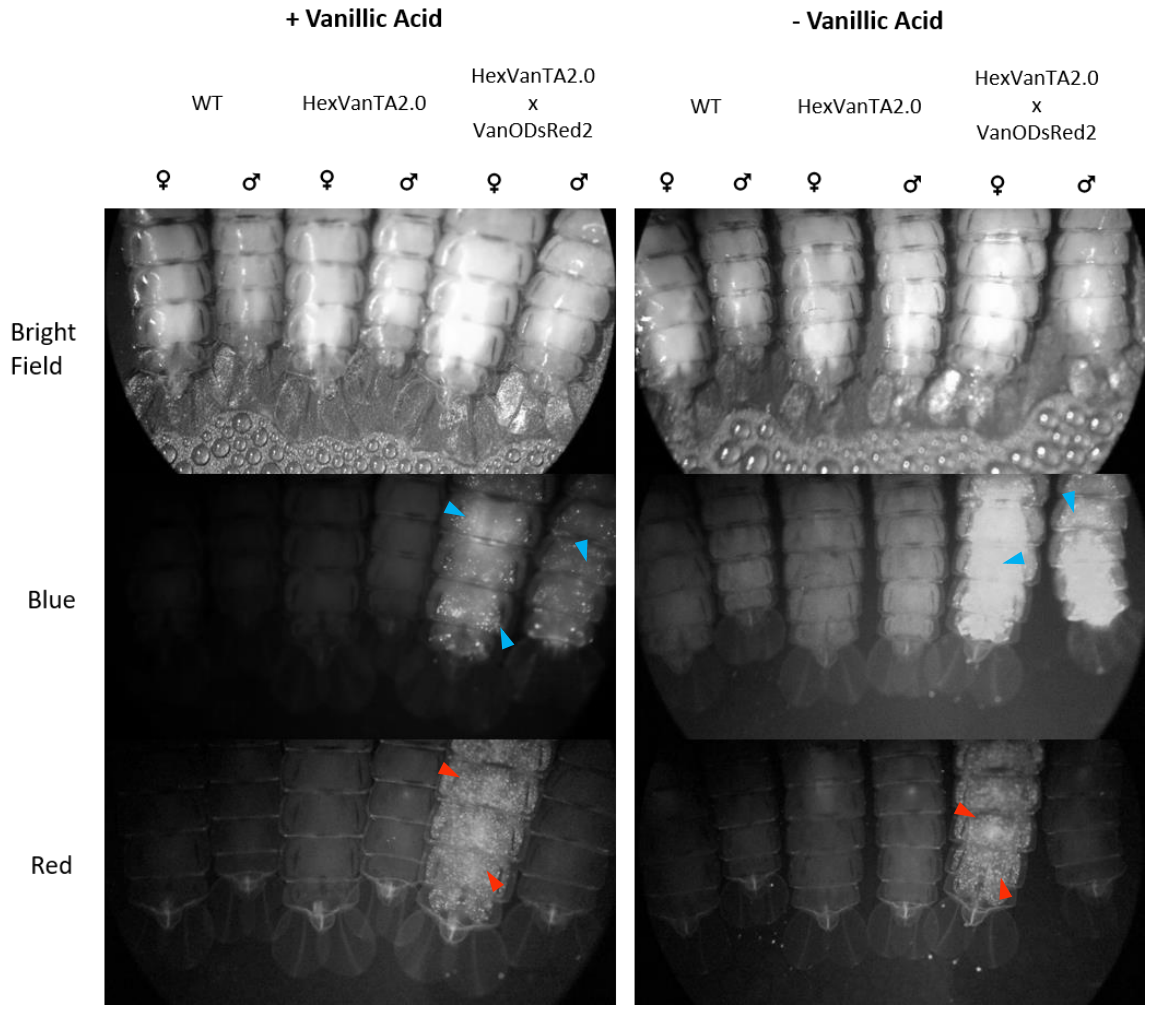


Figure 3.20

Results of an on/off vanillic acid assay: the ventral tail section of progeny from cross 13A-B are shown (HexVanTA2.0-13A crossed to VanODsRed2-B) under bright light, blue filter and red filter. Expression of Hexamerin driven DsRed2 was best seen in the tail of the pupae on the ventral side. The VanODsRed2 transformation marker (blue arrowheads) is clearly visible in both sexes and is not present in either of the control pairs, wild type and HexVanTA2.0. There is clear expression of the expected “red bodies” phenotype in female pupae carrying both constructs (red arrowheads), irrespective of rearing conditions. This is DsRed2 expression under a hexamerin promoter driven by the binding of VanR-VP16 to VanO. There is no visible expression of DsRed2 in males carrying both constructs irrespective of rearing conditions.

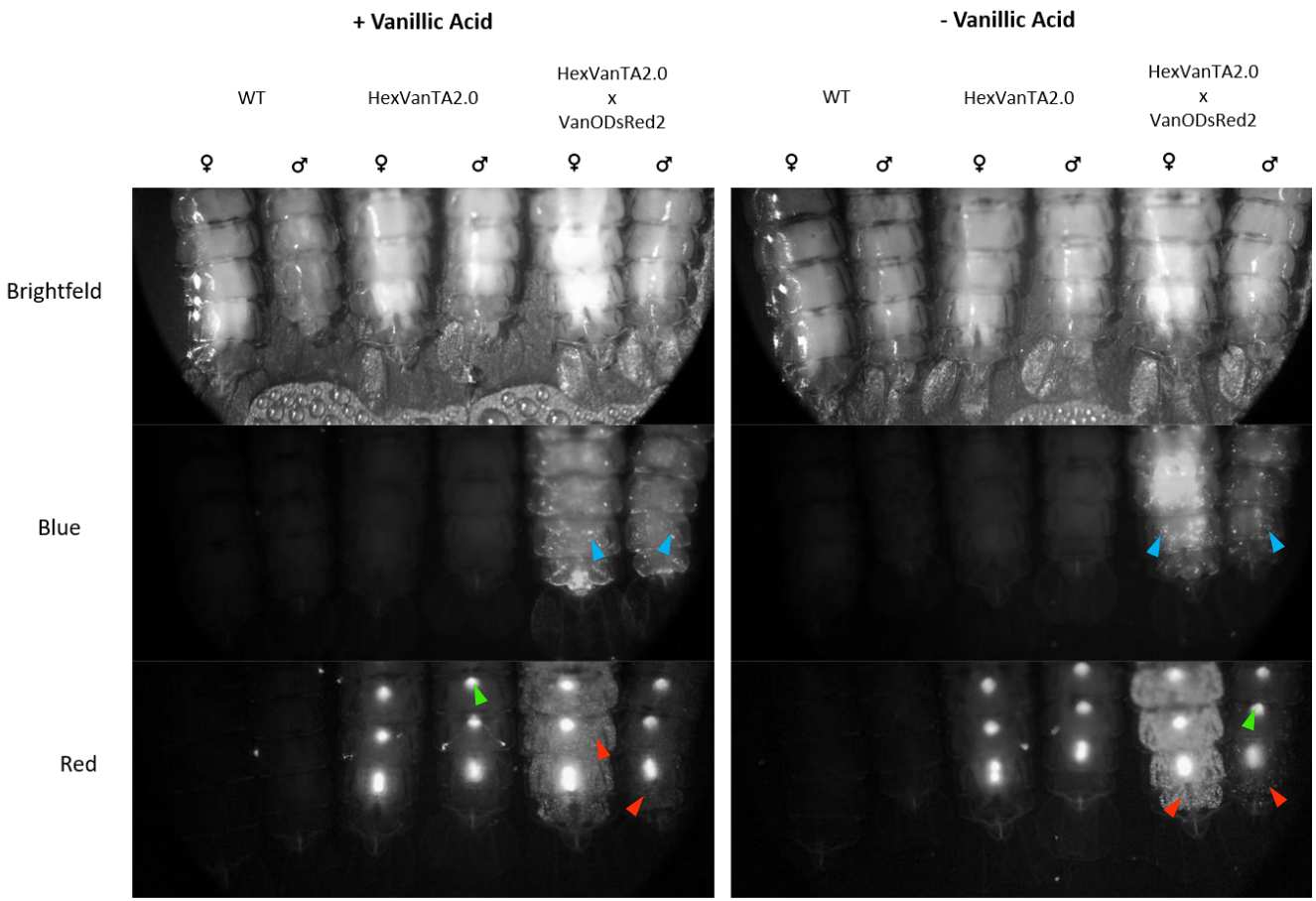


Figure 3.21

Results of on/off vanillic acid assay. Progeny from cross HexVanTA2.0-16A vs VanODsRed2-B are shown under bright light, red filter and blue filter. The VanODsRed2 marker can be seen when viewed under blue light (blue arrowheads). The HexVanTA2.0 transformation marker can be seen as bright discrete dots, one per segment (green arrowheads) and is clearly visible in both sexes. Hexamerin driven expression of DsRed2 can be seen irrespective of rearing condition (red arrows). Expression is brighter in female pupae compared to male pupae.

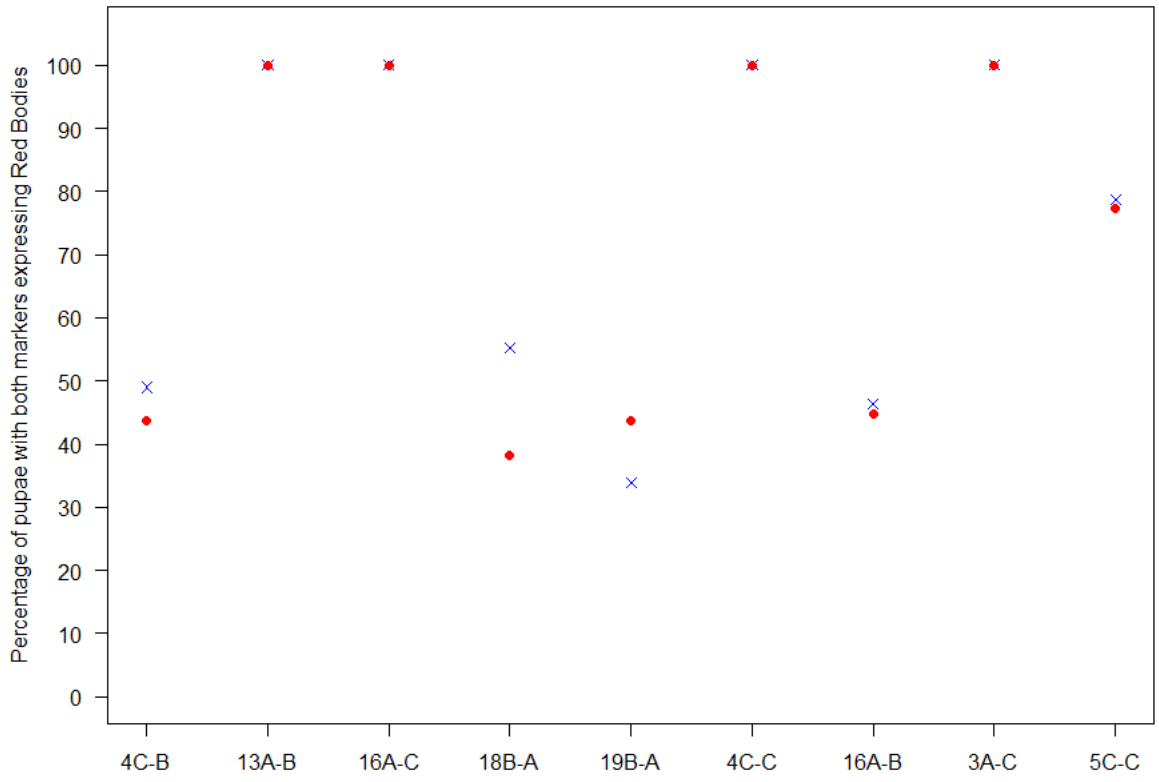


Figure 3.22

The percentage of pupae that carried both markers and expressed the Red Bodies phenotype for each cross when reared in water (blue cross) and when reared with vanillic acid (red dots). No significant difference was found between the number of individuals expressing red bodies when reared on and off vanillic acid for any of the crosses tested (Fisher's exact test).

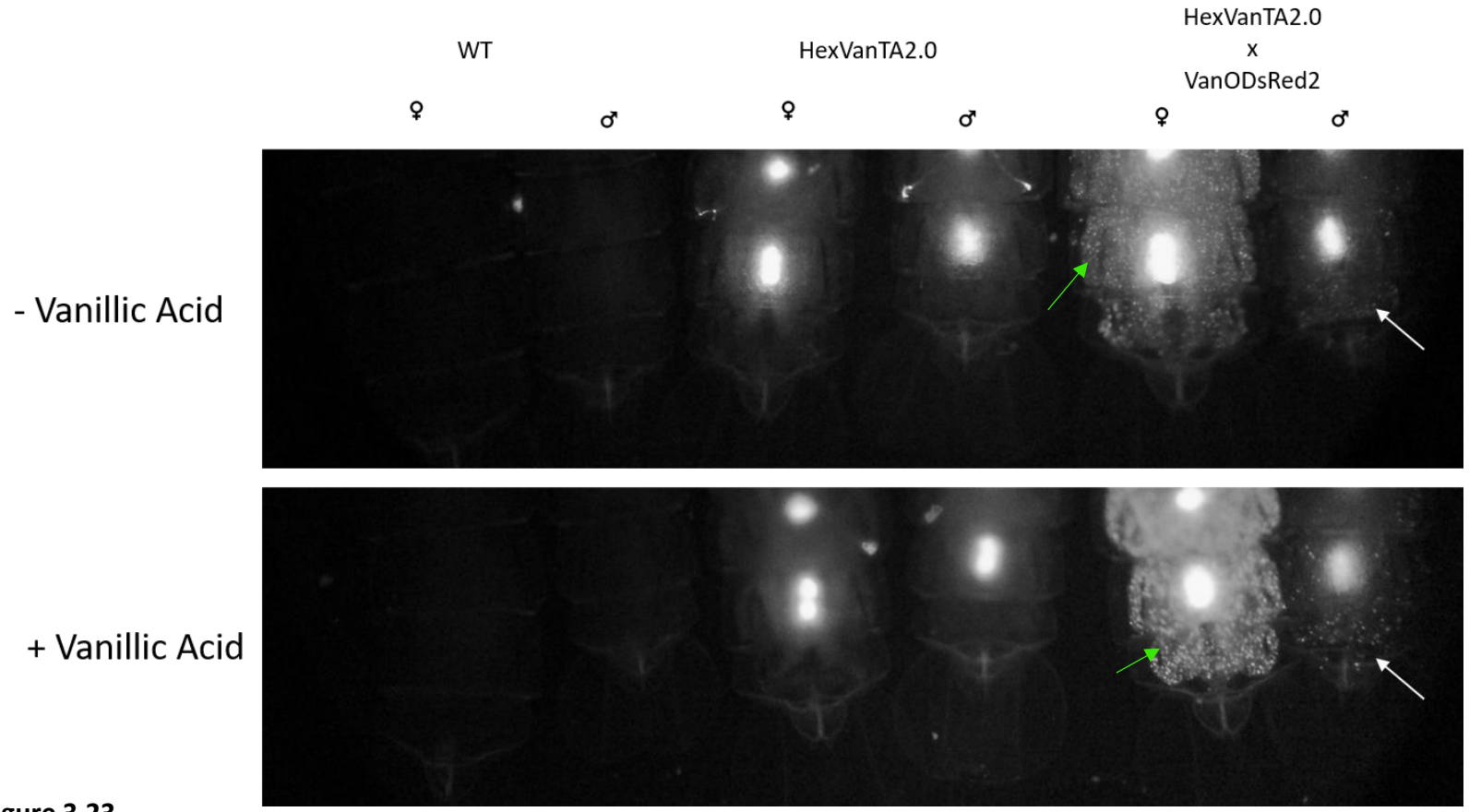


Figure 3.23

An enlargement of the HexVanTA2.0-16A vs VanODsRed2-B cross as seen under a red filter to highlight the difference in expression pattern for male and female pupae carrying both constructs: the trans-activator and the operator. The large bright dots are part of the 3xP3 marker cassette and stem from the 'red eyes' marker phenotype. Males do show expression of Hexamerin driven DsRed2 (white arrows) this is clearly reduced in males compared to females (green arrows).

3.4 Summary of Outcomes

The intended outcome for this chapter was to engineer a vanillic acid responsive genetic switch in the mosquito species *Aedes aegypti*. Several stages were completed including:

- The generation of transgenic strains carrying either the operator sequence or the trans-activator sequence

- Presence of the transgenes does not negatively influence survival of mosquitoes to pupation

- Progeny resulting from crosses of the transgenic lines expressed DsRed2 in the expected expression pattern. Demonstrating, that the fusion protein VanR-VP16 can be translated within *Aedes aegypti* and that it can subsequently bind the VanO sequence to drive targeted gene expression.

As it stands this system does not currently respond to the presence of vanillic acid and therefore expression cannot be silenced by the addition of the food additive to the rearing water.

3.5 Discussion

Transgene control systems in the mosquito *A. aegypti* form the basis for a genetic method of wild population control. There is a requirement for species control as *A. aegypti* is a principal vector for several globally important diseases. Currently a tetracycline responsive switch is used to control lethality in juvenile mosquitos however it would be beneficial to have an additional orthogonal control ligand. This would allow for multiple selection processes to be controlled genetically though the simple addition of a dietary supplement. Vanillic acid serves as a good alternative to tetracycline, it is a common food additive that is readily available and the system has been demonstrated to work in bacterial systems, mammalian cells and in transgenic mice.

Several constructs were built and injected into *Aedes* eggs to create a functional vanillic acid responsive system in the dengue fever mosquito. Individuals carrying VanODsRed2 and, either HexVanTA2.0 or HexVanTA2.1 showed the expected expression pattern of the marker. This expression was not seen in the controls. This demonstrates that the trans-activator is being transcribed and is efficiently binding to the operator sequence driving the expression of the fluorescent protein. The system tested here was not repressible as the addition of vanillic acid to the rearing water did not affect the phenotype of the mosquitoes. There was also a clearly visible difference in the expression pattern between males and females carrying the two constructs.

Hexamerins or hexameric storage proteins are the most abundant protein reserves carried over from the larval to the pupal stages in holometabolous insects. These proteins are stored in the fat body of the insect (Gordadze *et al.* 2003; Zakharkin *et al.* 1997). The pupal stage of the *Aedes* lifecycle is non-feeding, whilst the insect is undergoing metamorphosis, the stored hexamerins provide a source of amino acids for metabolism and protein synthesis. In *Aedes aegypti* there are two hexamerin proteins; hexamerin 1 (Hex-1) and hexamerin 2 (Hex-2). Hex-2 is present in the haemolymph of fourth instar larvae and early pupae and builds steadily to a peak just after pupation, there is no difference in expression between males and females. In comparison Hex-1 is largely absent from the haemolymph however it accumulates quickly in the fat body following the pupal moult (Totten *et al.* 2013). Previous studies have shown that Hex-1 demonstrates sexual dimorphism with females expressing at much higher levels than males, although there is still detectable expression of Hex-1 in males (Gordadze *et al.* 2003; Totten *et al.* 2013). The 5'-flanking region cloned by *Zakharkin et al.* (2001) from the mosquito *Aedes atropalpus* has been shown as sufficient to drive expression of hexamerins in *Aedes aegypti*. The promoter sequence used in both HexVanTA2.0 and HexVanTA2.1 is identical to the sequence

identified by *Zakharkin et al.* (2001). It is unsurprising then that during this study there was dimorphic expression of the reporter gene as expression of endogenous Hex-1 is dimorphic showing biased expression in females. In future studies an alternate promoter should be considered as Hex-1 is not sex-specific so cannot be used to target one sex over the other. If the same expression profile is desired with high expression in larvae and early pupae then the 5' region of Hex-2 could be used instead.

There are several explanations as to why the system was not repressible by the addition of vanillic acid. The concentration of vanillic acid in the rearing water may have been too low and thus there was not enough vanillic acid present in the fat body cells to cause repression. In this study the final concentration of vanillic acid in the rearing water was 0.3 mM. Unpublished data collected after this study by Amandine Collado performed the assay with concentrations of 10 mM vanillic acid in the rearing water. This had a significant effect on survival and had no effect on the observed phenotype. An alternate feeding method was also tested where vanillic acid powder and 3g of food were mixed into an agar solution. This was then set on petri dishes and portioned into cubes. Three vanillic acid cube concentrations were tested: 2.4 mM, 24 mM and 240 mM. The food cakes had no discernible effect on survival however once again there was no detectable change in phenotype between individuals reared with feeding cubes containing vanillic acid and control feeding cubes.

Alternatively, the trans-activator may have too high an affinity for the operator sequence *in vivo* this could be addressed by mutating the vanillic responsive repressor protein (VanR) and identifying a mutant that has a lower affinity for the operator site. The sequence used in this study is taken directly from the *Caulobacter cresentus* genome. Another study in 1994 looked at *Enterococcus faecium* and the DNA-binding site for the VanR protein that contributed to vancomycin resistance. They found that by changing an Asp53 residue to alanine the VanR still bound to the DNA but with significantly less affinity (R. Holman *et al.* 1994). This is most likely because phosphorylation of VanR is inhibited. A similar point mutation could be used here. To accelerate the process, the system could be tested in *Drosophila* which has a shorter generation time than *Aedes aegypti* or even tried in insect reporter cell lines.

In conclusion this work has demonstrated that the VanTA and VanO elements are sufficient to drive expression of transgenic genes in *Aedes aegypti* and this would likely transfer across to other insect species. These two elements would be kept in separate lines and then crossed to allow activation of the transgene. There is scope to make this a small molecule repressible switch that could operate in insect species.

4.0 Concluding Remarks and Future Work

The auxin responsive system components tested here did not vary considerably from the successfully executed system published by Trost *et al.* (2016). This system is functional in *Drosophila melanogaster* and will make a valuable addition to the current repertoire of tools available to developmental researchers. In the future the system sequences used by Trost could be combined with the conditional sterility application described in Chapter 2.

The vanillic acid system tested here did turn on demonstrating that the core components for the Van-Off system are being produced and that the VanR-VP16 fusion protein is folding properly and recognises and binds to the operator system. This shows that the system is viable for use in insect species. In the future the binding site of the VanR protein could be modified to limit its affinity for the operator thus making the system more responsive to vanillic acid. To expedite this process the system could first be trialled in *Drosophila* cell systems before being translated back into whole organisms.

Though further work and refinement is required these two systems will provide an additional level of control when discerning gene function in developmental biology, they are also suitable for application to industrial uses.

Going forwards starting using cell lines rather than whole organisms will shorten the time between iterations of the construct allowing for a functional system to be developed more readily. Trost *et al.* (2016) began by transfected S2 cells and viewing them using fluorescent imaging and taking time-lapse photographs to show that the auxin was entering the cells and affecting the system by turning off expression. A similar construct could be designed for the Vanillic acid system. Once the system has been established in S2 cells then it can be moved up to whole organism. The generation time in *Drosophila* is shorter than in *Aedes aegypti* therefore it makes more sense to establish the system in *Drosophila* first.

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Acknowledgements

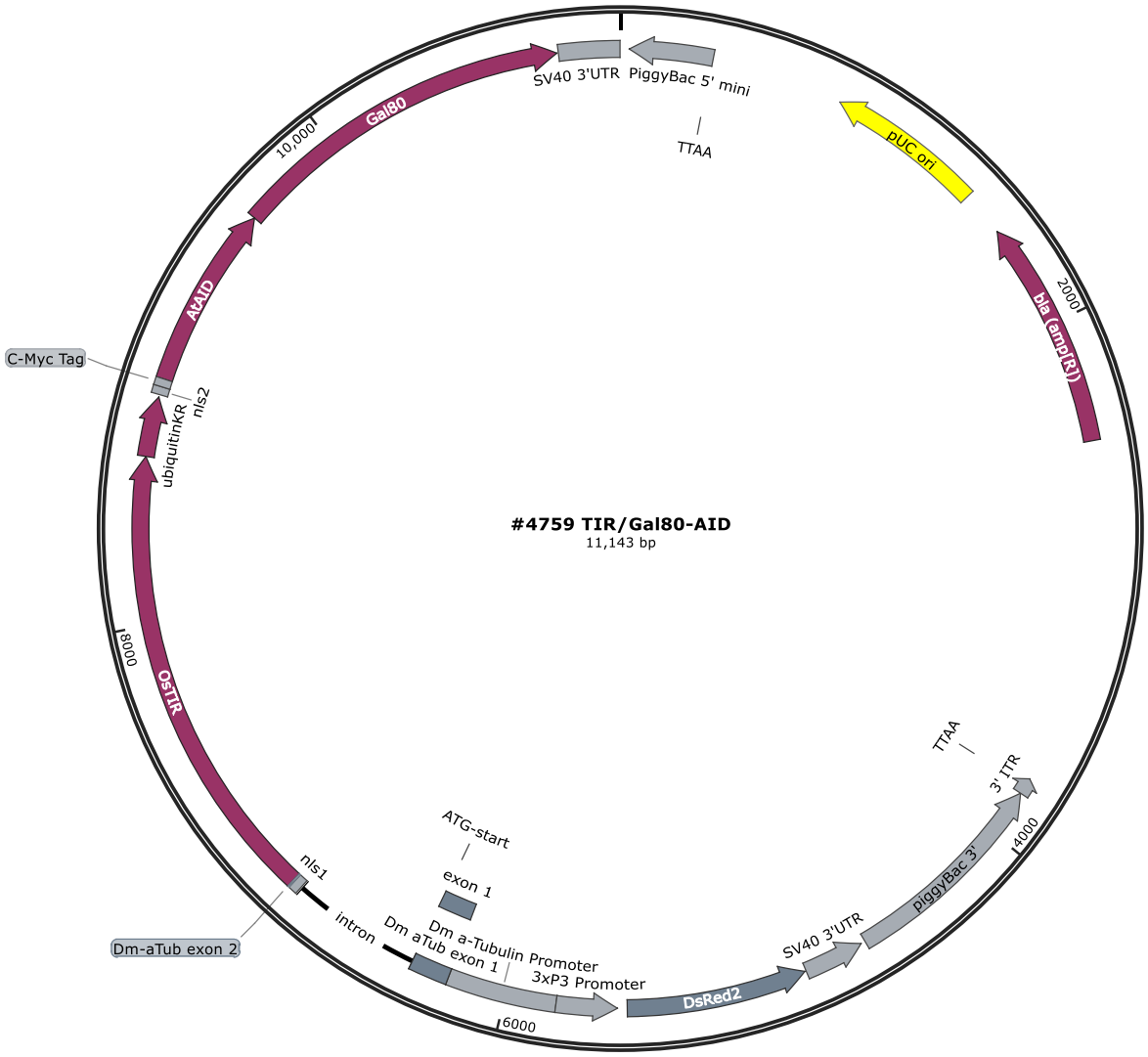
This work would not have been completed if not for the unwavering support and belief of my supervisor Prof. Helen White-Cooper. I also owe a lot to my friends, my sister, my cousin, my dad and my partner who believed that I could get this done. Thank you.

Thank you to the BBSRC for funding my work and to Oxitec for hosting me and sharing their knowledge and skills with me.

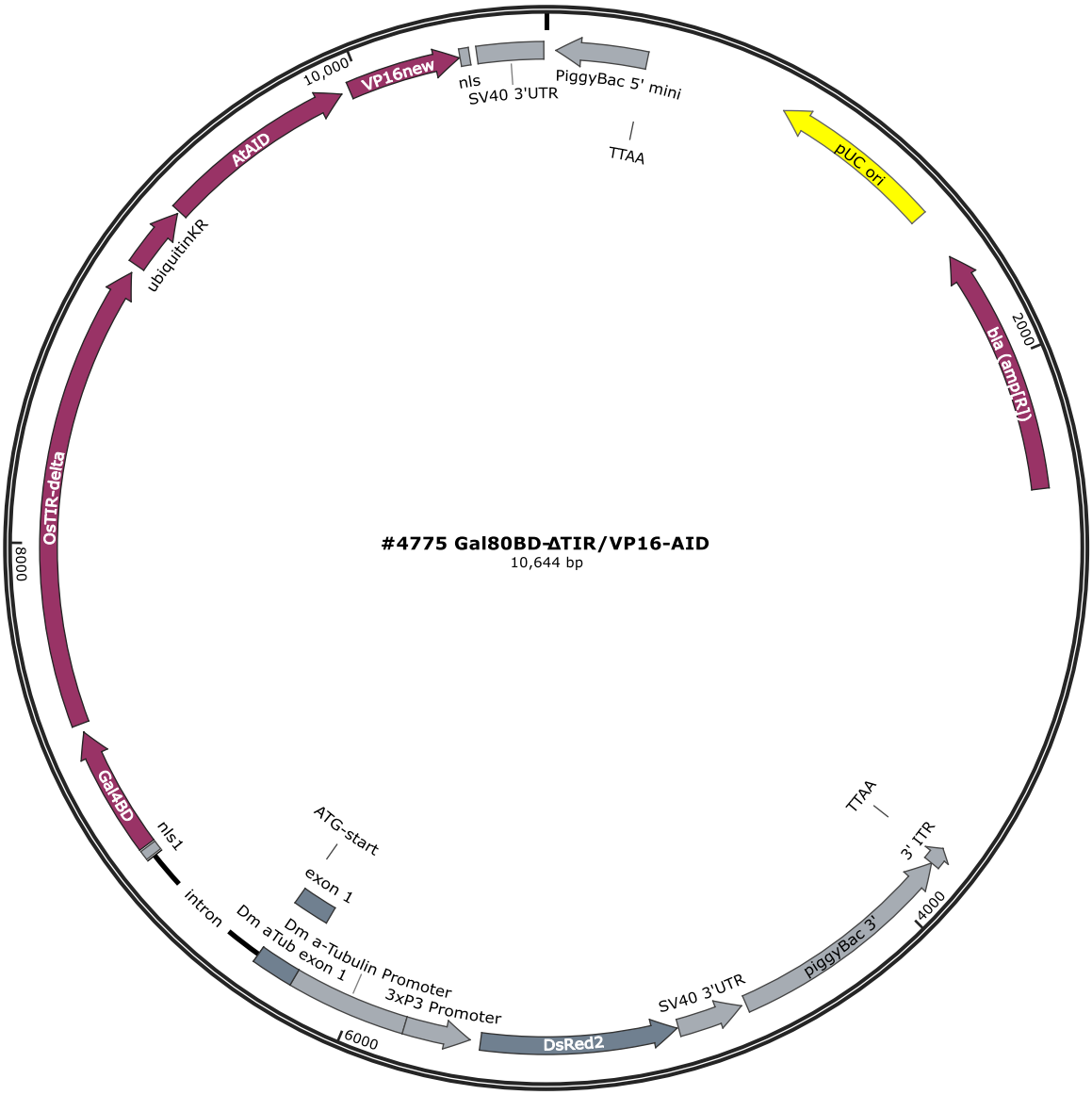
Appendices

Appendix 1: Auxin switch system construct maps

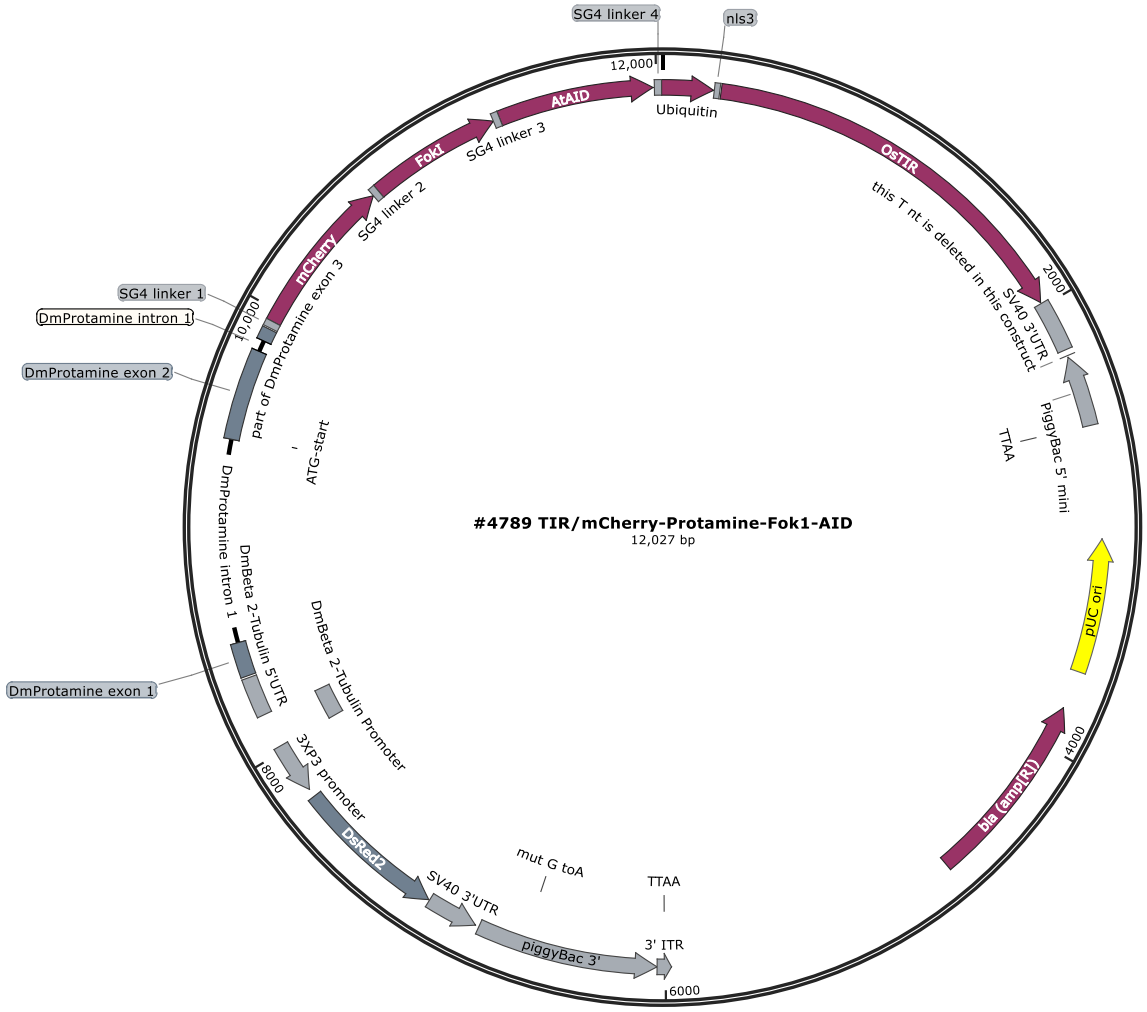
#4759: TIR/Gal80-AID



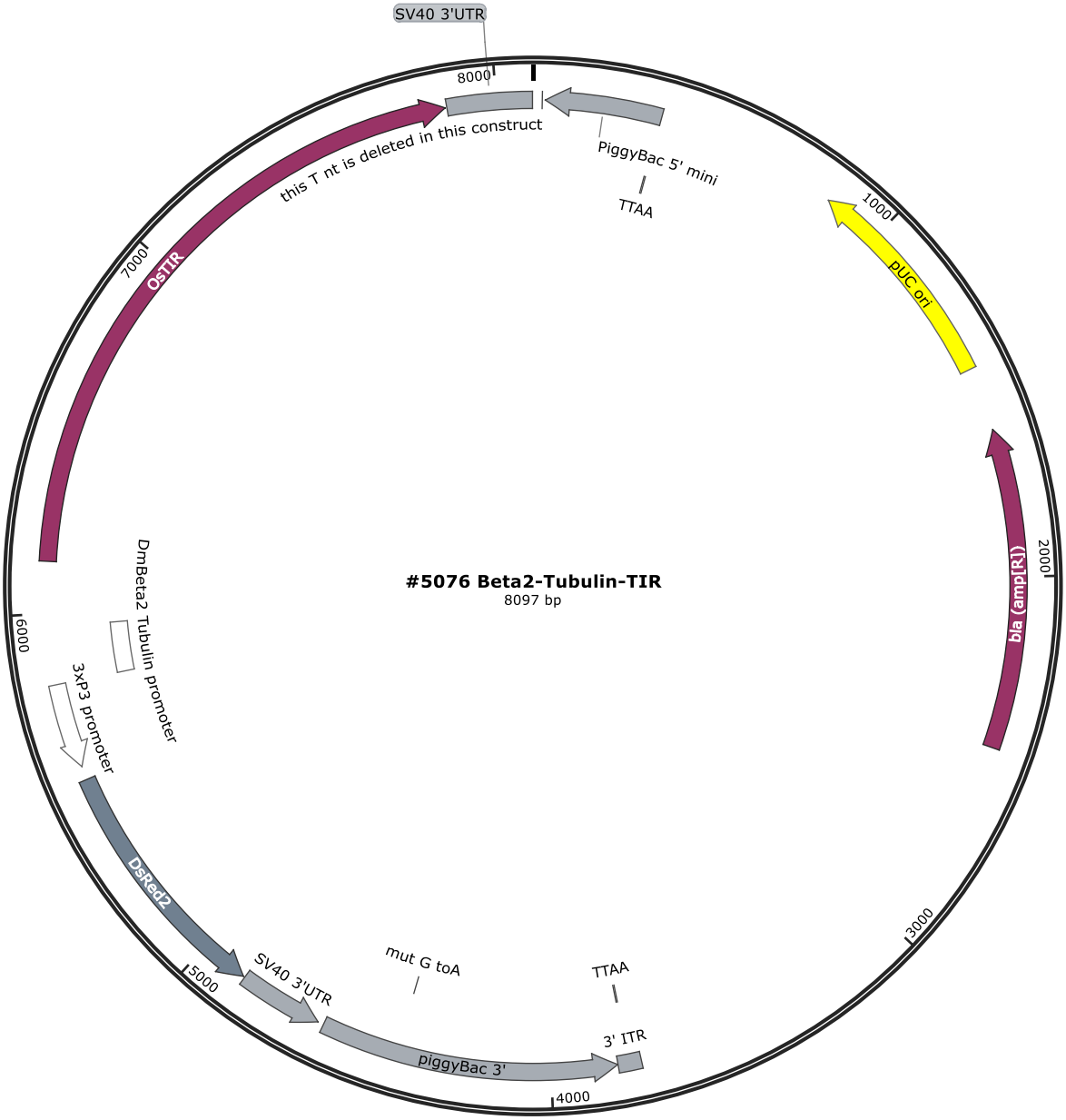
#4775: Gal80-BD-ΔTIR/VP16-AID



#4789: TIR/mCherry-Protamine-Fok1-AID



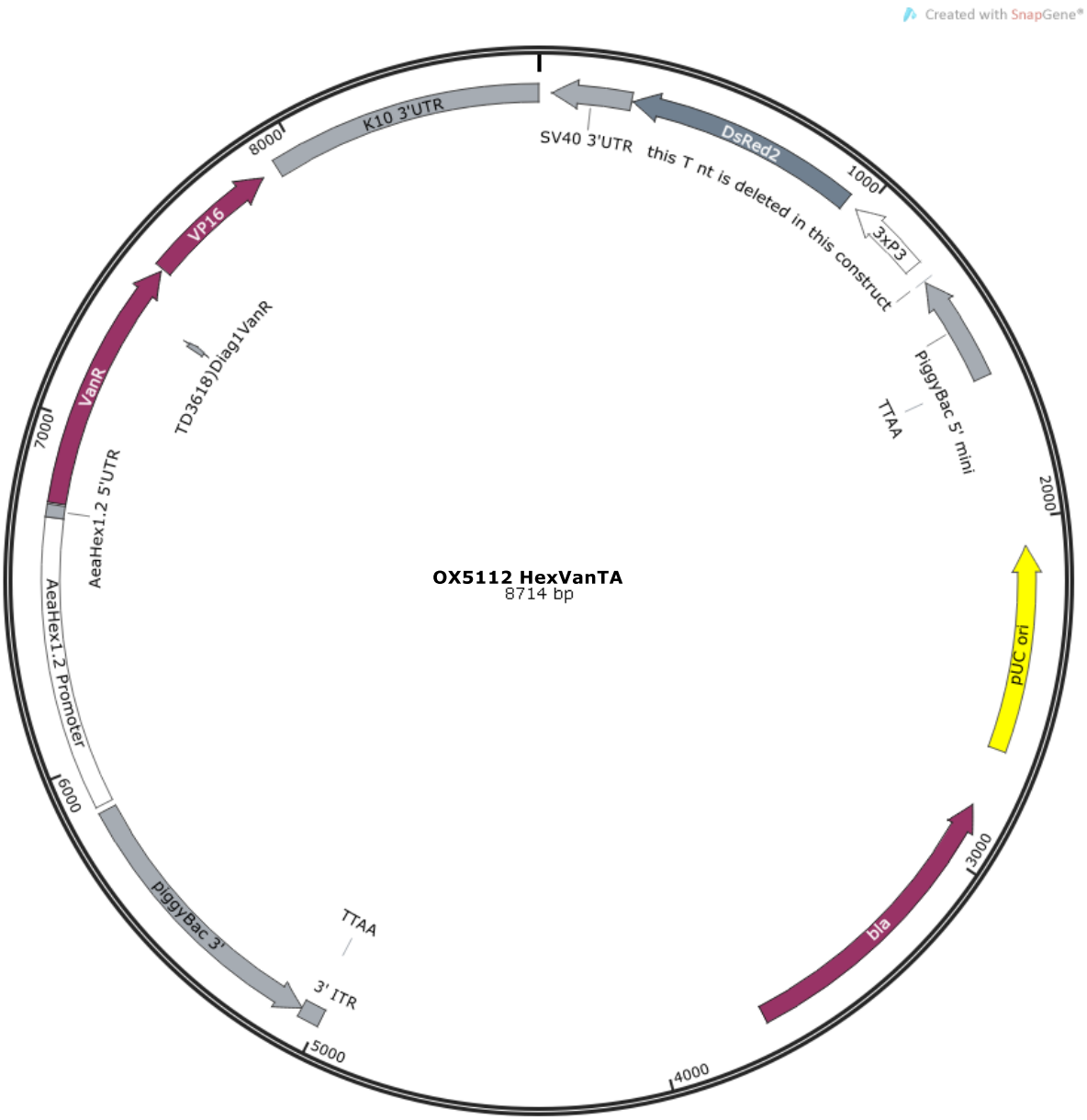
#5076: β2-Tubulin-TIR



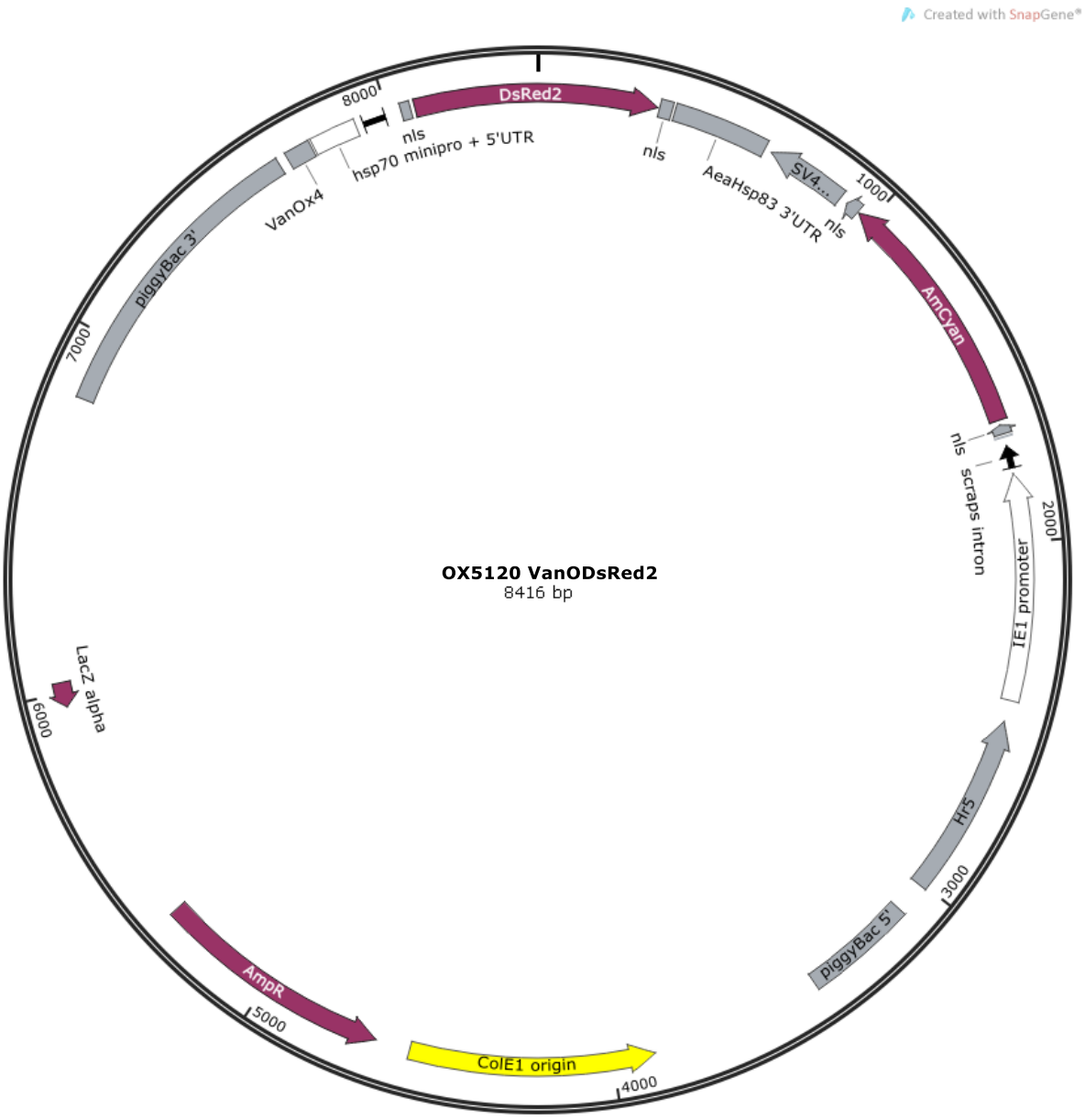
#5085: Fok1-AID

Appendix 2: Vanillic acid switch system construct maps

#5112: HexVanTA



#5120: VanODsRed2



#5161: HexVanTA2.0 and #7178: HexVanTA2.1

These two constructs only vary from each other only in terms of VanR2-VP16 sequence – translation and thus the expected protein products are the same.