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Engineered genetic sterility of pest insects

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Declaration of Authenticity

This work was supported by Biotechnology and Biological Sciences Research Council *(*BBSRC) studentship and Oxitec Ltd. I hereby declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. None of this work has been submitted previously for a qualification at the University of Oxford or another institute of higher education.

Abstract

In the light of increasing pesticides resistance in agricultural pests and in insect vectors of human diseases, leading to the rise in occurrence of mosquito-borne diseases, new, efficient and environmentally friendly methods of pest control are needed.

Sterile Insect Technique (SIT), relying on mass releases of radiation sterilised males to reduce reproductive potential of target pest populations, although not new, offers an alternative to the use of pesticides and is an environmentally non-polluting method of insect control. Many insect species, however, are not very amenable to classical SIT, due to detrimental side-effects of radiation treatment.

We propose a new method, a genetically engineered modification of classical SIT, replacing radiation with genetically induced sterility. Based on conditional expression of male-germline targeted nucleases which introduce double strand breaks into the male germline DNA to render males sterile, this method emulates SIT mechanism, at the same time eliminating radiation and associated detrimental side-effects.

Different variants of such a system were investigated in this project, eventually leading to the creation of functional conditional male-sterility systems in two model organisms – the Yellow fever mosquito, *Aedes aegypti* and the Mediterranean fruit fly, *Ceratitis capitata.* Both systems utilise chimeric nuclease composed of protamine and FokI cleavage domain fusion. The sperm-specificity and the conditionality of the sterile phenotype have been achieved through the use of tetracycline repressible expression system driven by the β2-tubulin promoter in *Ceratitis capitata* and by the Topi promoter in *Aedes aegypti*.

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Chapter 1 - Introduction

1.1. Insects as pests and disease vectors

The word "**pest"** means "a plant or animal detrimental to humans or human concerns (as agriculture or livestock production)" [Miriam-Webster dictionary]. A broader meaning of this term covers also disease-spreading animals, especially when they are responsible for epidemic diseases associated with high mortality among affected species.

Although insects are highly beneficial to humans, many of their species are also considered major pests due to the effect they have on our economy, agriculture or health. The damage that insects cause may be direct, by feeding on crop plants or stored products, or indirect, by spreading diseases of crop plants, livestock animals and humans themselves.

The majority of insect pests belong to the order *Diptera* (true flies). Their characteristic feature is a presence of only one pair of flight wings and so called "halters" – balancing organs that evolved from the second – hind – pair of wings. Dipteran insects undergo complete metamorphosis (holometabolism), with a life cycle that comprises an egg, larvae, pupae and adult stage of development. There are two suborders of *Diptera*: the *Neumatocera* – which have multi-segmented antennae and long, thin bodies and the *Brachycera* – which have short stylate antennae and thick bodies [90].

In the UK alone there are over 6200 species of *Diptera* [90]. Approximately 200 species world-wide are recognised as pests. Crop plants pests include: the

Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), the boll weevil, *Anthonomus grandis* (Boh) and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Among livestock pests are such species as the New World Screwworm, *Cochliomyia hominivorax* (Coquerel) and the Blowfly, *Lucilia sericata* (Meigen), whose larvae feed on living tissue. Examples of human disease vectors among *Diptera* are the mosquito species *Anopheles gambiae* (main vector of malaria in Africa), mosquito *Aedes egypti* that spreads the dengue fever, chikungunya and yellow fever viruses, and the Tsetse fly, *Glossina morsitans* (Westwood), which carries trypanosomes causing human sleeping sickness and animal trypanosomiasis (nagana disease).

Two insect species that belong to order *Diptera*, were used as model organisms for the genetic sterility system in this project: the Mediterranean fruit fly, *Ceratitis capitata,* and the Yellow fever mosquito, *Aedes aegypti*.

1.1.1. Mediterranean fruit fly – *Ceratitis capitata*

Ceratitis capitata [*Fig. 1.1*] is a true fly from the family *Tephritidae* of the suborder *Brachycera*. It diverged from *Drosophilidea* (family which the most common fruit fly, *Drosophila melanogaster,* belongs to) about 80-100m million years ago [14]. Native to the Mediterranean, it is now widely spread around the world on all continents, apart from more northern and arctic regions. Such a wide distribution was possible because of its higher tolerance for cooler climate than other tropical fruit flies, and its ability to infest a wide range of host plants (several hundred). These characteristics make *Ceratitis capitata* the most destructive agricultural pest in the world, and much effort is put into controlling this species [40].

Mediterranean fruit flies are holometabolous insects (undergoing complete metamorphosis). Under optimal conditions, the female can lay around 300 eggs during her lifetime. Eggs are laid under the skin of the host plant fruit which scars the fruit. Eggs hatch within 3-5 days into larvae, which feed in the flesh of the fruit for the next 6-10 days – until reaching third, final instar. Medfly larvae are highly mobile and may "jump" between fruits. Fully-grown larvae leave the fruit and drop onto the ground, where they borrow into the soil to pupate. Adults emerge 7-10 days later and require another 3-5 days to reach sexual maturity. Adult flies typically live between 20-30 days in optimal conditions [82].

Costs of Medfly damage are large and are due to a destruction of fruits or vegetables infested with flies. Oviposition and feeding on fruit flesh are the direct cause of damage but scaring plant tissue may also lead to secondary infections from invading bacteria and fungi that would cause rot. Larvae can also attack young seedlings, tap roots, stems and buds of host plants. In addition, high economic costs are inflicted due to quarantine and monitoring programs, limits on export from fly infested areas, and quarantine treatments of fruit from infested areas [121].

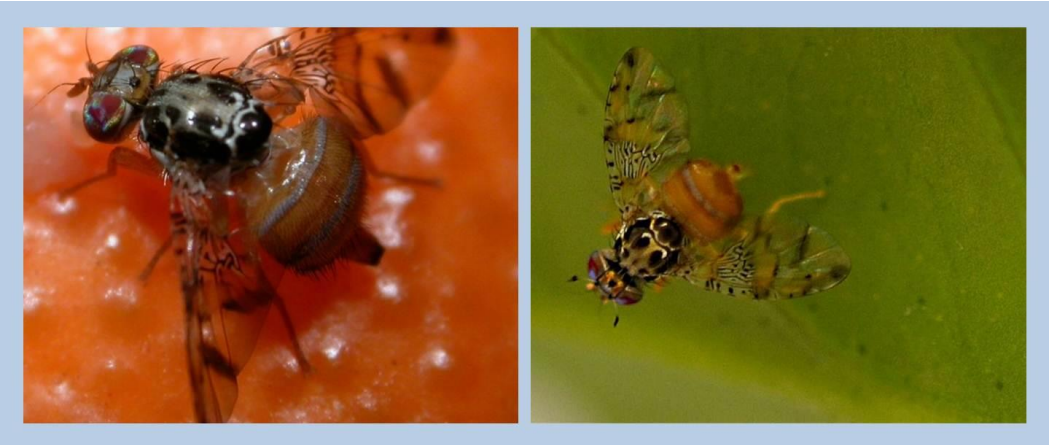


Figure 1.1. Adult Mediterranean fruit flies. Left – female; right – male. Female ovipositor and bulbed-shaped male antennae are visible. Image courtesy of Oxitec Ltd.

1.1.2. Yellow fever mosquito – *Aedes aegypti*

Aedes aegypti is a mosquito from the family *Culicidae* of *Diptera* order. It is a vector insect transmitting several tropical diseases, including Chikungunya and Yellow fever in humans and Rift Valley fever that primarily affects livestock but may spread onto humans who are in direct contact with infected blood. It is also a major vector of dengue fever and its more severe form – haemorrhaging dengue fever, and is responsible for the recent worldwide spread of this disease [43].

Dengue fever is endemic to most tropical countries and many sub-tropical regions (presently estimated to be endemic to over 100 countries) but imported cases were identified also in non-endemic countries [24]. An outbreak of dengue on Madeira Islands in 2012, affected 1800 people and imported cases were detected in five other countries in Europe apart from mainland Portugal. According to WHO estimates, up to 40% of the world population may be presently at risk. There may be 50–100 million dengue infections worldwide every year. In some Asian and Latin American countries, severe dengue is a leading cause of serious illness and death among children [WHO, 2013]. No effective vaccine for the treatment of dengue fever has been developed to date.

Yellow fever affects African and American countries of the tropical regions. Although it can be fatal (when patients enter so called “toxic phase”), most patients improve with time. The disease causes serious outbreaks but their occurrence can be controlled by preventive vaccinations [WHO 2013]. Unfortunately, only a few African countries reach the required level of vaccination to fully protect their populations.

The word “Chikungunya” means: “that which bends up’’ and very well describes the posture of patients experiencing severe joint pains that are a characteristic feature of this disease. The disease is present in Africa, Southeast Asia and India. In 2007, the first cases were reported in north-eastern Italy showing that Chikungunya fever has already spread into Europe [27, 28]. The only available treatment is the administration of fluids and analgesics [WHO 2013].

Aedes aegypti is very well adapted to life in man-made habitats, like garden pots filled with water, discarded car tyres etc., virtually any habitat providing stagnant water. This feature, in conjunction with feeding almost exclusively on humans and being active throughout the day, has made this species a very efficient dengue virus vector [36, 43].

Only adult female mosquitoes bite, requiring a blood meal to support production of eggs. Without access to blood, adult females feed on plant nectar, the same as adult males. Upon blood feeding, the female can lay between 100 to 200 eggs, although the number depends on the size of the blood meal. Eggs are laid in the moist areas within close proximity of water likely to flood the eggs. Once submerged in water, eggs hatch producing larvae, the aquatic stage of the mosquito life cycle. Mosquito larvae feed on organic matter found in the water, and after the fourth instar, enter the pupal stage, 7-10 days from hatching. Atypically for holometabolous insects, a mosquito pupa is a mobile, although not feeding, stage. After about 2 days, adult mosquitoes [*Fig 1.2*] emerge, 2-3 days later reaching sexual maturity.

Mosquito-spread diseases cause millions of deaths every year, primarily in the developing countries to which they are endemic. Recent reoccurrence of large numbers of disease incidents are due to human population growth and the development of insecticide and drug resistance among insects. Migration

(including travelling) and climate change (warmer conditions) allow for rapid increase in the geographical ranges of the disease that used to be considered endemic to smaller regions [1, 45].

Figure 1.2. **Adults of Yellow fever mosquito *Aedes aegypti***. Left – female; right – male. Image courtesy of Oxitec Ltd.

Several approaches may be undertaken to try to control mosquito-borne disease. This may be achieved by eliminating a vector, introducing protection against mosquito bites (insect repellents, bed nets) or direct action against a disease (vaccination).

1.2. Traditional methods of pest insects control

Traditional insect control methods can be classified into a few categories:

(1) Cultural control – avoiding pests or trying to make an environment less favourable for a pest. This includes elimination of breeding grounds like stagnant water in the case of *A. aegypti*. However, control programmes based on this method require a proper education campaign and

coordinated community effort [135]. In poor areas or regions that rely mainly on stored rain water, it is very difficult to deliver the close control of breeding sites necessary for this method to be successful in reducing the numbers of disease-transmitting adult mosquitoes [9].

(2) Host resistance – concerns plants and uses the natural ability (physical and chemical adaptations) of some of the plant species to repel, tolerate, or even kill pests. It has been used by plant breeders for decades. However, the main limitation in the use of this method in modern agriculture is the adaptability of pests and pathogens that can overcome the resistance [107].

(3) Physical control – relies on keeping pests physically away from their host, like introducing barriers (window or crop netting, wearing appropriate clothing etc.) or setting insect traps. These methods are not equally effective against all insects and largely depend on the behaviour of the pest.

(4) Mechanical control – direct removing or killing the pest (like hand-picking or dislodging with water spray, etc.). These methods are quick and effective and, moreover, they have relatively little impact on the environment and other non-target organisms but they are not suitable for large-scale pest problems.

(5) Biological control – employs natural enemies of the pest (predators, parasites or pathogens) to control its population. These methods may be highly successful, like the use of parasitic wasps to control Tephritid fruit flies [6]. Unfortunately, they may also prove disastrous as it was in a case of the cane toad *Bufo marinus,* which was introduced to Australia in 1935 to eradicate grey backed cane beetles. The toad "ignored" the beetles as a

potential prey and instead started hunting native species of insects and small amphibians. It is also poisonous for humans and animals [http://www.environment.gov.au/biodiversity/threatened/ktp/cane-toads.html].

(6) Chemical control – uses natural or synthetic chemicals to kill pests or to inhibit their natural behaviours like feeding or mating. Chemicals may work as repellents, confusants, and irritants that usually are not toxic to insects, but interfere with their normal behaviour (mosquito repellents or moth balls]. Toxic chemicals aimed at killing insects are called insecticides. Synthetic organic insecticides have been developed for nearly every insect pest. They are effective, cheap to produce and to apply, and have been used in agriculture as well as in the battle against disease-carrying insects. Their effects, in general, are predictable and reliable. Reliable, however, in "killing insects" and not necessarily in "killing only selected insects". The main disadvantage of insecticides is that they are not highly selective and most can affect non-target organisms. Very often insecticides are highly toxic to beneficial insects, such as pollinators and predatory or parasitic natural enemies. Insecticides can be also hazardous to humans and pet or agricultural animals. An additional disadvantage is that both target and non-target insects can develop resistance to insecticides, sometimes very rapidly [78].

1.3. The Sterile Insect Technique

As mentioned above, an increasing occurrence of pesticide resistance in pest populations due to pesticide exposure is a major problem. On top of the environmental damage, it is one of the major challenges facing modern agriculture and programmes aimed at controlling disease-transmitting insects. An attractive alternative to the use of pesticides is the biological pest control relying on predation, parasitism or other natural mechanisms – one such a method is the Sterile Insect Technique (SIT). It was pioneered in the 1950s by American entomologists Raymond C. Bushland and Edward F. Knipling who jointly received the 1992 World Food Prize for their achievement.

SIT involves releasing millions of sterilised insects of the target species over a wide area to mate with the native insects, causing a reduction in the proportion of fertile matings in the wild population. First, insects must be mass reared. Sterility is induced through X-rays, gamma radiation or chemosterilants that cause chromosomal aberrations and dominant lethal mutations in the sperm [16]. Sterile insects are then released into the wild population and the sterilised males compete with wild males for matings with wild females. Mating of released sterile males with native females leads to a decrease in the females' reproductive potential because their offspring do not survive. Ultimately, if males are released in sufficient numbers over a sufficient period, this leads to the local elimination or suppression of the pest population [70]. The necessary number of sterilised males that can affect the wild population depends on the biotic (reproductive) potential (R_0) of this population. Over-flooding with sterile males is required. For example, to reduce wild population with five-fold biotic potential to 50% of its initial

density, the effective over-flooding sterile male ratio must be 9:1. Successful releases should decrease biotic potential of wild pest population to below 1.0, decreasing its numbers with every generation. This, however, requires continuous releasing of sterilised insects per each generation. Large programmes may continue for many years. The New World screwworm programme took more than 40 years before it managed to eliminate the infestation in North and Central American and establish a barrier zone in Panama [23].

SIT has many advantages; it is species-specific and has no effect on other 'non-target' species, hence it is environmentally clean and sustainable. The target species does not need to be monogamous but SIT programmes must deal with a possible impact of immigrating fertilised females from the regions that are not covered by similar programmes. The success of SIT programmes largely depends on a good understanding of the biology and ecology of a target species, and seasonal patterns of its distribution and density in given area. Wild population reduction will depend on the competitiveness of the released insects, on the frequency and distribution of the releases, longevity of sterilised individuals and their ability to locate mates, and on predation [23]. The classical SIT programme suffers from other problems. The released insects are required to compete for mates with the wild insects. The production process, however, and in particular, the need to sterilise the insects by irradiation, causes a dramatic loss of competitive mating ability relative to the wild type. Irradiated insects are less competitive and also have reduced life span. In Medfly, the combination of these two factors leads to an estimated reduction in fitness of 4–10-fold [74, 112]. This deficiency in fitness must be compensated for by the increased numbers of released insects. In addition, it is best to release only male insects as females can

have detrimental effects. Although being sterile, released females may attempt to oviposit, or obtain a blood meal, and so cause the type of damage that the control program is intended to overcome. In some cases it is possible in the laboratory to separate males and females by criteria such as pupal mass or time of eclosion, but these methods rarely yield a truly single-sex population. Existing genetic sexing mechanisms based on classical genetics (chromosome aberrations) are not perfect either – they tend to reduce overall viability of insects carrying them, are not stable and must be developed anew for each new species [2].

However, it is worth mentioning here that there exists a common confusion in the literature, where a term "SIT" is used instead of "Female Killing (FK)". FK is a genetic strategy that employs autosomal insecticide-resistance genes pseudolinked to the Y-chromosome and in *trans* with multiple deleterious recessive mutations. To separate two genders, insecticides were used in the laboratory conditions to kill females (males would be resistant). After releasing males into the wild population, the load of recessive mutations, accumulated with time, would kill any homozygous female offspring. Generating FK system involves introduction of chromosomal translocations and employs irradiation. The analyses of several failed FK trial programmes (conducted mainly with *L. Cuprina*), have proven that there is a fundamental and irreversible cost of irradiation needed to generate targeted chromosomal rearrangements. In addition, a decrease in male fitness appeared to be linked to a number of FK elements introduced into their genome [16].

The sterile insect technique was applied to control many other insect pest species. Tsetse fly (*Glossina* sp.) is successfully controlled and one of its species, *Glossina austeni* (Newstead) was eradicated from Unguja Island in Zanzibar in 1997,

eliminating economically significant infections in local cattle [30]. The largest tsetse mass-rearing facility in the world, the Tsetse and Trypanosomiasis Research Institute in Tanga, Tanzania releases up to 70,000 sterile males per week. Around the world, there are now about 20 fruit fly rearing facilities, one of which is capable of producing over three billion sterile male *Ceratitis capitata* pupae weekly for commercial purposes. However, to date, no successful "operational level" mosquito SIT programme has been conducted [23].

1.4. Genetic engineering of insects

Like many other life sciences fields, insect control was revolutionized by the advent of recombinant DNA technologies, which have armed scientist with new tools in their fight against pest insects. One of the most important new methods was the ability to genetically transform the insects. The real turning point in transgenesis of many insect species, especially of economic importance, came with the discovery of the piggyBac transposon. Discovered in the cabbage looper moth, *Trichoplusia ni,* by Fraser *et al.* [32] piggyBac is a 2472 bp class II mobile element that transposes via the cut-and-paste mechanism as a result of enzymatic activity of transposase encoded in its sequence. It exclusively inserts itself into the TTAA sites, through the asymmetric cut leading to duplication of TTAA site upon insertion, and returning the insertion site to its original state after remobilisation [15].

PiggyBac consists of two terminal repeat sequences flanking a 2.1 kb transcriptional unit containing the piggyBac transposase ORF. Each of the repeats

is composed of a 13 bp perfect inverted terminal repeat (ITR) followed by a 19 bp subterminal repeat and it is necessary for transposase binding during remobilisation [47].

A bipartite vector-helper system derived from the piggyBac transposon has been utilised for use in the germline transformation of insects. In such a system, the central fragment containing a transposase transcriptional unit is replaced by the transgene intended to be integrated into the host genome. The transposase, which is necessary for integration, is supplied separately. This can be done in the form of a helper plasmid encoding transposase under the control of an early embryonic promoter, transposase mRNA derived from such plasmid, or as a purified protein. The piggyBac-based germline transformation system proved to be very versatile and capable of transforming various species of insects, spanning three orders [47, 49].

The use of the piggyBac transposon would not have become so successful and widespread without the development of new transformation markers. Classical genetic marker systems usually rely on the rescue of a mutant phenotype. In such systems, a phenotypically easily identifiable dominant allele masks expression of a recessive mutant allele, when they occur in the same genome together. This can happen as a result of an integration of a transgene "marked" with the dominant allele into the genome containing the recessive allele or as a result of crossing between two lines carrying either recessive or dominant allele. One example of such a marker would be the well-known *white eye* mutant allele from *Drosophila melanogaster* or its Mediterranean fruit fly equivalent [137].

Such markers, unfortunately, have to be created anew in each species, which is a laborious and not always fruitful process. To expand the use of piggyBac, or other

germline transformation methods for that matter, beyond *Drosophila melanogaster* or *Ceratitis capitata*, fluorescent proteins are being used, as much more universal markers. They can be detected at varying expression levels, are dominant and are suitable for use in wild type organisms, a feature crucial for the applications in pest management programs [55]. Typically used fluorescent proteins are Green Fluorescent Protein (and its blue variant AmCyan), isolated originally from jellyfish, *Aequorea victoria* [115], and DsRed, from the sea anemone, *Discosoma striata* [81].

To take advantage of these characteristics of fluorescent proteins as transformation markers, appropriate promoters have to be available. Such promoters have to be constitutive, active in most tissue types (or at least in the easily visible ones) and at the appropriate stage of the insect's life cycle. Examples of such commonly used promoters include: ubiquitin, actin, 3xP3 or hr5IE1. Both 3xP3 and hr5IE1 promoters were extensively used during this project.

The 3xP3 is an artificial, eye-specific promoter derived from the binding site of Pax 6 / Eyeless – a transcriptional activator controlling development of the eyes in metazoan animals [56, 113]. The hr5-IE1 promoter is composed of the baculovirus enhancer (hr5) and the immediate-early promoter elements [114]. Examples of expression patterns of DsRed fluorescent protein driven by these promoters in transgenic *Aedes aegypti* lines are shown in *Figure 1.3.*

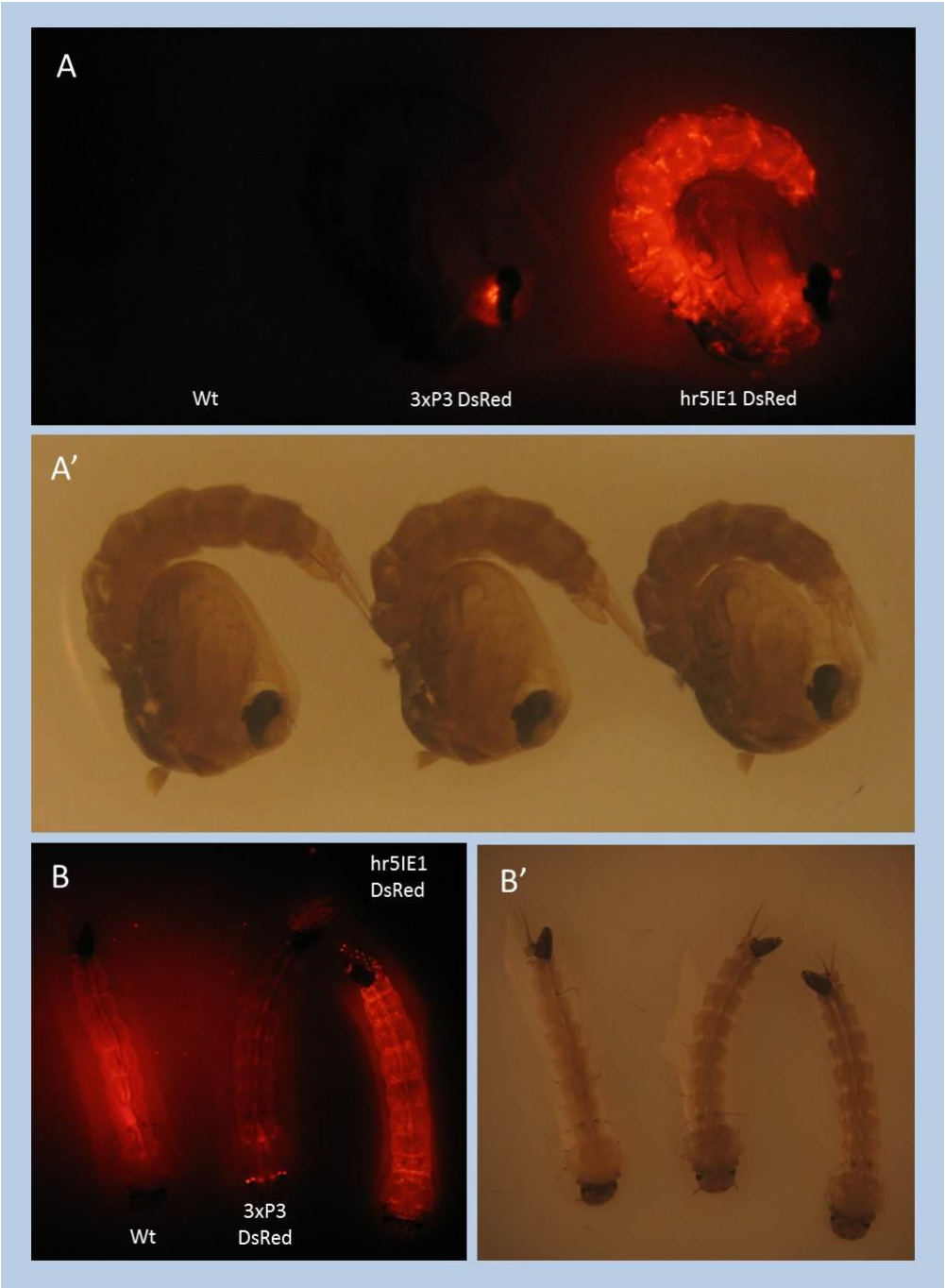


Figure 1.3 Examples of fluorescent protein marker driven by different promoters. Images showing spatial patterns of expression of the DsRed fluorescent protein marker in *Aedes aegypti* pupae (A) and larvae (B) driven by 3xP3 promoter (OX4286-B-Aae line) or hr5IE1 promoter (OX3979-Aae line).

1.5. RIDL technology

Drawing on advances in insect transgenesis, RIDL technology (Release of Insects carrying Dominant Lethal) - a modification of SIT technique - was developed. It uses the conditional, tetracycline regulatable gene expression systems and overcomes many of the classical SIT problems mentioned in the previous chapters.

RIDL relies on a conditional expression of a lethal effector that kills insects carrying it. Mechanistically the system comprises tetracycline-repressible transcriptional activator (tTA or tTAV) driven by a desirable promoter (for example female specific one) and a lethal effector, the expression of which is under the control of tTA response element (tetO). The promoter used to drive expression of tTA activator defines gender and developmental specificity of the system. All components are combined onto a transposon used as a vehicle to integrate the whole system into the insect genome. Tetracycline-repressibility of tTA confers conditionality of the system switching it on or off in the absence or presence of the repressor (tetracycline) [2, 120] [*Fig 1.4*].

A progeny of the transgenic insects carrying the RIDL system (female progeny for instance, to continue with above example) will die in the absence of the repressor (tetracycline). While under the laboratory conditions insects can be easily reared with a dietary supplement of tetracycline, the lack of a repressor in the wild will activate the lethal system. Released transformed males, homozygous for the lethal construct, would pass one copy of the dominant lethal to their offspring that would subsequently die as larvae or pupae in the wild due to the absence of tetracycline.

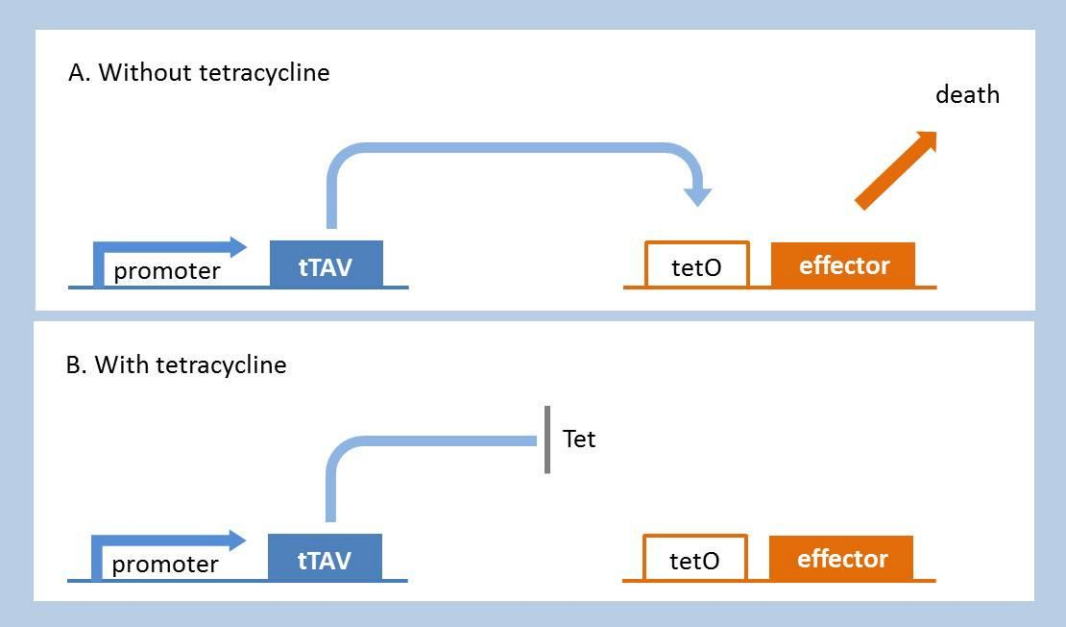


Figure 1.4. **The tetracycline-repressible expression system in RIDL.** The transcriptional activator (tTA) protein coding sequence is placed under the control of the promoter of choice. When expressed, the tTA protein binds to tTA response element (tetO) driving expression of the lethal effector – **A**. However in the presence of tetracycline tTA does not bind to tetO and expression of the effector gene is prevented – **B**.

Releasing RIDL insects in sufficient numbers leads to an increased proportion of non-viable progeny and has a similar population-dumping effect as SIT. The RIDL system offers the following advantages:

- By replacing irradiation, it minimises the damage done to the insects; RIDL insects are fitter compared to the radiation sterilised ones. Recent experiments with RIDL olive fly strain and RIDL *Aedes aegypti* strain have shown that the fitness of engineered RIDL males is comparable to that of the wild type strain [3, 80]
- It provides a genetic sexing mechanism (if female lethal effectors are used) allowing male-only releases;

- It has in-built fail-safe properties – due to the lack of access to the repressor outside of the laboratory / rearing facility;
- It is fully species - specific and it uses insects' own mate-seeking behaviour what allows for a precise and effective targeting a chosen population;

1.6. How to make a sterile male and why (benefits of DNA double-strand breaks)

Broadly defined sterility, the inability of an insect to produce viable offspring, can result from several factors:

- female infecundity – when females are unable to produce and lay eggs,
- inability of either sex to mate,
- aspermia – when males fail to produce spermatozoa,
- sperm inactivation – inability of the sperm to function properly, for example when the sperm are immotile,
- dominant lethal mutations in the genetic material of either the sperm or the egg;

Sterile Insect Technique relies predominantly on the last of these methods. Dominant lethal mutations represent true genetic sterility, in that the affected males are able to produce viable sperm, which are transferred to the females during mating and are able to fertilize the eggs, but development of the formed zygote does not progress beyond early stages. Radiation induced dominant lethal

mutations are caused by the double-stranded brakes in the chromosomes. When occurring in mature sperm, such breaks are not repaired and resulting fragments remain separated until the fusion of the sperm and egg nuclei following fertilization. Separated fragments of the chromosomes lacking centromere are eventually lost during nuclear divisions, causing serious genetic imbalances leading to cellular and eventually zygote death [117]. A diagram, depicting this mechanism is shown in *Figure 1.5*.

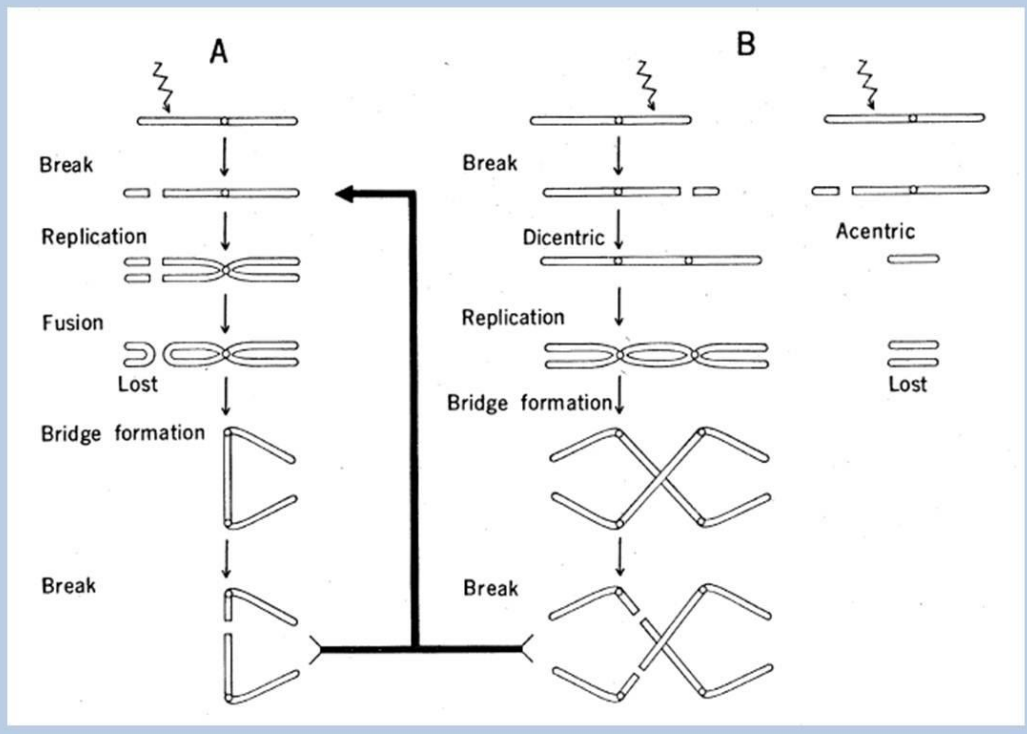


Figure 1.5. **Schematic representation of molecular mechanism of lethality caused by DNA double-strand breaks**. Because double-strand breaks in the mature sperm DNA are not repaired, chromosome fragments remain separated. After fertilization, fragments lacking centromere are lost during nuclear divisions in the zygote leading to its eventual death. Effects of DSBs in a single chromosome (**A),** and in two non-homologous chromosomes (**B**). (Figure from [117])

Irradiation-induced sterilisation used in classical SIT programmes, damages insects, compromising their performance and lifespan, and poses environmental

and health and safety hazards. However, the fact that it works by introduction of double strand breaks into the DNA can be advantageous.

The RIDL system relies on lethal genes, which can be disrupted by the DNA mutations. There is also a possibility of wild insects developing resistance to the lethal effector or, for example, evolving some sort of antidote / inhibitor to tTAV protein. Introduction of the multiple Double Strand Breaks (DSBs) into DNA damages genetic information beyond repair, making the development of resistance virtually impossible – it is difficult to see how the maternal or zygotic genotype could compensate for the damage that has already been done to the sperm before it enters the egg.

A conditional genetic sterilisation system which utilises endonucleases to introduce double strand breaks into the insects' germline DNA represents an alternative to both classical SIT and RIDL methods. Based on the same sterility mechanisms as radiation sterilisation, such a system has the potential to lower the risk of resistance problems, comparing to RIDL, at the same time offering the same advantages as RIDL:

- an on/off switch via a dietary additive (tetracycline) allowing mass-rearing and easy induction of sterility;
- very specific, localised effect – minimising damage to the insects;
- being more cost effective than irradiation;

Finally, unlike the RIDL insects, genetically modified insects carrying nuclease-based sterilisation systems are true sterile insects, a feature that could be beneficial with respect to the regulations concerning releases of genetically modified organisms.

1.7. Conditional genetic sterility system

In their simplest form, genetic sterilisation systems can consist of a DNA nuclease under the control of a sperm-specific promoter which would directly drive the expression of a nuclease. In fact such a system has been built by Windbichler *et al.* [131]. In their system they used I-PpoI homing endonuclease, which selectively targets X-linked *Anopheles gambiae* 28S ribosomal genes, with the intention of inducing sex ratio distortion in transgenic mosquitoes.

Sperm-specific expression of GFP-labelled I-PpoI was achieved by utilising β2-tubulin promoter. I-PpoI successfully cleaved 28S ribosomal genes on the X-chromosome resulting in early embryonic lethality of embryos fertilized by transgenic males. This difference in phenotype, comparing to the intended one, was most likely due to carry over of I-PpoI into the zygote where it targeted X chromosome from the egg. It was, nevertheless, the first demonstration of transgene-induced male sterility in mosquitoes.

In such a system, however, male sterility is non-repressible. It is not possible to turn the nuclease off which means that males are always sterile. Transgenic lines have to be propagated through crossing of transgenic females to wild type males since it is not possible to obtain true-breeding homozygous lines.

Conditional expression systems eliminate these problems as they allow turning the nuclease effector on or off and hence repressing the sterile phenotype in transgenic males. Conditional genetic sterility system can be composed of the elements described in *Figure 1.2* – sperm-specific promoter driving expression of tetracycline-repressible trans-activator and DNA nuclease under the control of the

tetO operator. But what kind of promoter and what kind of nuclease effector would be the best?

1.7.1. Spermatogenesis and germline promoters

From the perspective of our project, one of the most important features of spermatogenesis is a transcriptional shut down or meiotic arrest. The development of mature sperm cells proceeds in the germline from diploid spermatogonia, through primary spermatocytes (diploid), secondary spermatocytes (haploid) to spermatids (haploid) and finally, individualized, mature spermatozoa (haploid). Primary spermatocytes are transcriptionally active but in the later stages, with the onset of the first meiotic division, most transcripts become transcriptionally repressed. Transcripts required in the later stages of spermatogenesis, after meiosis, are transcribed pre-meiotically, stored as mRNA and translated later, when needed [37]. This transcriptional activation prior to meiosis is orchestrated by the so-called meiotic arrest genes, for example matotopetli (topi) [93].

This feature of spermatogenesis poses important constraints on the design of the sterility system described here, when it comes to choosing the promoters used to drive each element of the system. For example, tetracycline-repressible transcriptional transactivator driven by such a promoter, that it would be translated post-meiotically, may be unable to drive the expression of the nuclease through the tetO operator. For the expression of tTAV, a promoter of a gene with pre-meiotic function would be more suitable. On the other hand, the expression of the nuclease, and DNA cleavage, should not occur too early in spermatogenesis since this could lead to damage or loss of the germline, resulting in the production

of fewer or no gametes at all. Sterile males in the SIT program should be as competitive against wild type males as possible. Ideally they should be able to successfully copulate with the female, deposit the sperm and even fertilize the eggs, in terms of delivering sperm's genetic information, to mimic as much as possible the behaviour / performance of the wild type males.

Two promoters were considered as the main candidates for driving tTAV expression in the pest insects' male germline: β2-tubulin and matotopetli (Topi).

β2-tubulin is a highly conserved, extensively studied gene expressing testis-specific protein involved in many aspects of spermatogenesis. It is a major component of the sperm tail axoneme. β2-tubulin synthesis starts during late third larval instar before the onset of meiosis in the developing testis and continues throughout adult life of the insect [57, 116]. β2-tubulin promoters have already been utilised in different insect species, for example to drive expression of fluorescent sperm marker [110, 116] or to cause targeted cleavage of X chromosomes during spermatogenesis via the action of I-PpoI homing endonuclease in an attempt to distort sex ratios in *Anopheles gambiae* populations (both as improvements to the SIT technique).

Topi, encoding Zinc-finger transcription factor, belongs to the aly-class meiotic arrest genes; is essential for transcriptional activation of many testis-specific differentiation genes and is also involved in the regulation of cell cycle progression [93].

Many of the germline specific genes arose from duplication of their somatic counterparts, with a new copy acquiring a new, germline function – a good example being β2-tubulin [86]. In a lot of cases such duplications appear to be recent, hence in many insects both somatic and germline functions will be

performed by the single ancestral version, lacking separate somatic and germline promoters. This means it will be impossible to confine the expression of the effector to the germline using such promoters. Topi is different in that it is more ancient and does not seem to have a somatic version of the gene. Therefore, Topi derived promoter may function in the germline of wide range of insects.

Based on the *Drosophila melanogaster* Topi sequence, *Aedes aegypti* and *Ceratitis capitata* homologues were obtained in Oxitec, in collaboration with Dr Helen White-Cooper from Cardiff University. The conservation of expression in both species was confirmed. This was the starting point in the tests of suitability of this promoter for use in conditional male-sterile system.

1.7.2. Nuclease effectors

For the use in our proposed genetic sterility system, nucleases with many recognition sequences per genome, hence damaging DNA in multiple locations, would be preferable. This implies the use of endonucleases with short recognition sequences. Enzymes exhibiting a very high degree of sequence specificity and long recognition sequence will cut at very few sites per genome which allows the possibility of resistance by mutation or variation of the target site.

Another important feature of the nuclease is its requirement (or lack of it) for binding with other proteins for its activity – with itself as a homodimer (or homomultimer) or with different proteins in case of heterodimer (or heteromultimer). The key difference between dimerising and non-dimerising nucleases lies in the relationship between concentration and activity. The activity of the enzyme that does not need to dimerise will generally be proportional to its concentration. That means that even at very low concentrations there still will be

some detectable activity – and some DNA damage. In contrast, the enzyme that requires dimerisation will typically have a non-linear dose-response function. Especially in the case of the enzyme that can bind at many sites in the genome, there is little chance for two enzyme molecules to form a dimer and cut DNA at low concentrations. This forms an in-built protection against leaky expression of toxic effectors in the repressed state of the system – particularly in the untargeted cells / tissues. This is an important feature especially in the case of the conditional expression systems where even in the "off state" there is always some low level expression from minimal promoters. A schematic diagram depicting the difference between the types of nucleases discussed above is shown in *Figure 1.6*.

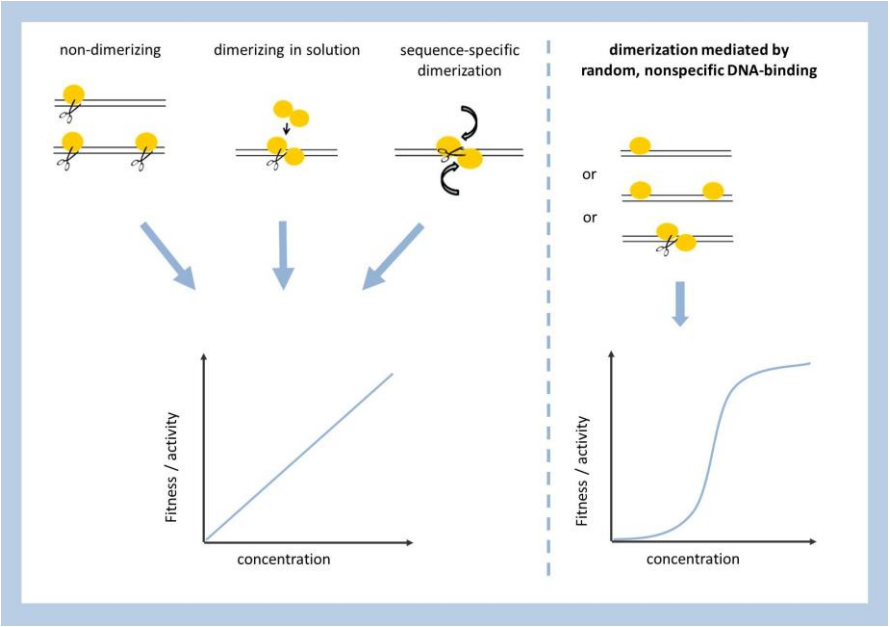


Figure 1.6. **The relationship between concentration and activity of different types of DNA nucleases.** Yellow ovals represent nuclease monomers, scissors icons indicate active state of nuclease, able to cleave double stranded DNA molecule. A linear relationship between nuclease concentration and activity means that even small quantities of nuclease – as in case of leaky expression in the repressed state of the system – can cause DNA damage. This may be detrimental for insects' fitness. A nuclease that forms an active dimer only when two monomers come into contact after DNA binding will have a sigmoidal dose-response profile. Especially in the case of the nuclease that can bind at many sites in the genome when there is little chance for two enzyme molecules to form a dimer and cut DNA at low concentrations.

Taking all of this into account, the nuclease that we proposed to use in our system is composed of a protamine fused to a FokI nuclease cleavage domain.

1.7.2.1. Protamines

Protamines are small, basic nuclear proteins that replace histones during post-meiotic stage of spermatogenesis. In the final stages of spermatogenesis spermatids mature into fully functional spermatozoa. DNA compaction in the sperm head is an essential part of this process and protamines play a crucial part in it. They bind the chromatin in a way that allows for better condensation and organization of DNA within the head of spermatozoon. This tight packaging of DNA affects head morphology and subsequently sperm performance and provides protection against DNA damage. [63, 64].

Protamines bind DNA through a series of small "anchoring" domains, each consisting of few arginines, which wrap around one loop of DNA, fitting into the major groove [Fig. 1.7]. One protamine molecule binds per turn of DNA helix. Cysteines, the second most abundantly present amino-acids, form disulphide bonds between neighbouring protamines. These covalent bonds bring protamines together, closely locking DNA strands. They also prevent a premature removal or dissociation of protamines from DNA before the sperm enters the egg [4].

During binding to DNA, protamines' positive net charge neutralizes the negative charge of the DNA phosphate backbone. This allows for very close packaging of DNA molecules and hence formations of much more condensed structure than formed through coiling of DNA around histones [4].

Vertebrate and invertebrate protamines have different amino-acid sequences, which suggests that their structure and function were evolving independently in different groups of animals All protamines, however, share one structural characteristic responsible for the ability to bind to the major DNA groove – the aforementioned abundance of positively charged arginine residues [4].

Two protamine transcripts were discovered in *Drosophila*: *Mst35Ba* and *Mst35Bb* (later called: dProtA and dProtB) whose sequences are similar to human protamines. They have almost identical amino-acid composition and are likely functionally redundant [64]. *Mst35Ba* and *Mst35Bb* are transcribed during the early primary spermatocyte stage, before meiosis. Their translation, however, is repressed until the post-meiotic canoe stage (elongated spermatids), when the functional protein is finally made [60].

The ability of protamines to stably and efficiently bind DNA during spermatogenesis makes them perfect candidates for sperm DNA targeting domains, specifically when binding in close proximity to each other is of crucial importance.

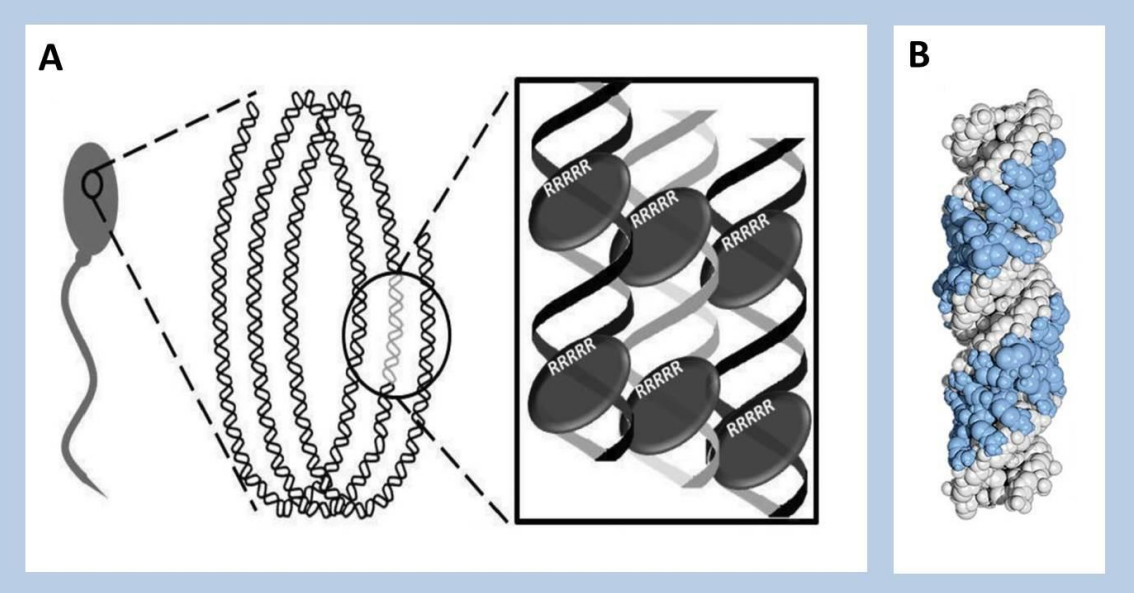


Figure 1.7. Chromatin condensation through protamine binding to DNA. **A** – Diagram depicting model of protamine binding to DNA and chromatin condensation. Protamines bind in the major groove of DNA molecule thanks to high arginine content. Positive net charge of protamines neutralizes the negative charge of the DNA phosphate backbone. Protamine binding results in DNA coiling into tight, toroid conformation. This structure is stabilised by formation of inter- and intra-molecular disulphide bridges between protamines cysteine residues (*image from [Kannipayoor 2013]*). **B** –Model showing two adjacent salmon protamine molecules (in blue) binding in the major groove of DNA helix (*image from [4]*).

1.7.2.2. FokI endonuclease

FokI is a restriction endonuclease discovered in the Gram-negative bacterium *Flavobacterium okeanocoites*, which lives in soil and fresh water [62].

Restriction endonucleases are ubiquitous among prokaryotic organisms. In conjunction with methyltransferases they form the restriction-modification (RM) system – bacterial protection against foreign DNA, mostly bacteriophage DNA. Both components of the system recognise the same specific sequences. In a strain of bacterium carrying the particular RM system all DNA sequences recognised by both enzymes are methylated by the methyltransferase protecting them from

cleavage by the endonuclease. Foreign DNA is not protected by this methylation and is degraded by the action of restriction endonuclease. Three main classes of RM systems have been found in bacteria, with members of type (class) II being of special importance due to their founding role in recombinant DNA technology [96]. FokI belongs to class IIs of restriction endonucleases. Typical type II restriction enzymes, like EcoRI, exist as homodimers in solution, usually recognising palindromic DNA sequences between 4 to 8 bp long. Upon binding to DNA as homodimers, they cut both strands of DNA – each monomer cutting one strand – with the cleavage occurring within the recognition site. FokI, on the other hand, exists in solution as a monomer and binds to the non-palindromic 5'GGATG-3' sequence cleaving DNA 9/13 nucleotides downstream of its recognition site [76].

Proteolytic and later mutational and structural studies showed that FokI consists of two distinct domains. The 41-kDa amino-terminal domain is responsible for sequence-specific DNA binding and the 25-kDa carboxy-terminal domain functions as a catalytic (cleavage) domain. The carboxy-terminal cleavage domain does not confer any sequence specificity. However, when purified on its own without the recognition domain, it was shown to be capable of non-specific DNA cleavage in *in vitro* assays [76, 15, 127, and 129].

Although existing as a monomer in solution, FokI has to dimerise for the double-stranded cleavage of DNA – FokI cleavage domain contains a single catalytic site. [15, 129] Dimerization is mediated through cleavage domains and requires the presence of specific DNA sequence and divalent magnesium ions. Prior to DNA binding, cleavage domain of the FokI monomer remains sequestered by intra-molecular interactions with DNA binding domain, kept in an inactive state, away from DNA. This sequestration probably helps to prevent any non-specific DNA

cleavage by FokI catalytic domain during the scanning of the DNA by FokI in search for the binding site. Upon finding its specific sequence, FokI recognition domain binds in the major groove of the DNA helix [136, 128]. Interactions between DNA and recognition domain lead to conformational change releasing cleavage domain from its sequestered state. Freed cleavage domain can come into contact with DNA by rotating around linker segment. To be able to cleave both DNA strands FokI cleavage domain has to form a dimer with the second cleavage domain. This second cleavage domain comes from different FokI nuclease bound to different recognition site elsewhere in the DNA, and it is activated in the same way – by release from the recognition domain upon DNA binding. The dimerisation interphase of FokI cleavage domains is rather small, hence weak – explaining monomeric state of FokI in solution – and the presence of magnesium ions is required to help with the formation of a dimer [15, 123, 127, 128]. Diagram depicting model of FokI-DNA complexes is shown in *Figure 1.8.*

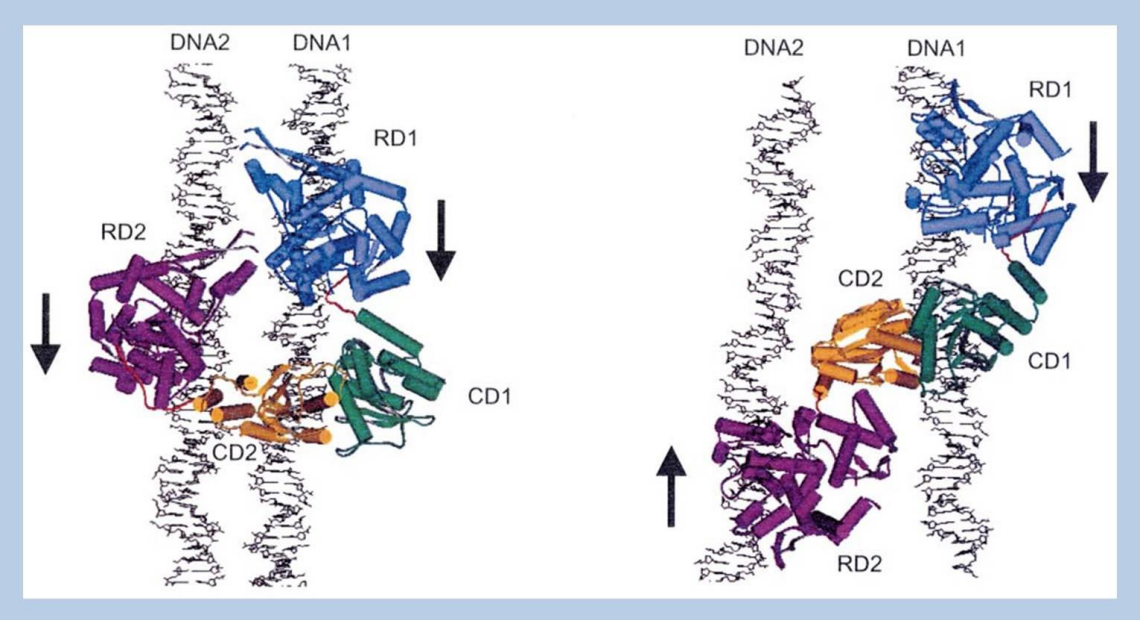


Figure 1.8. Models of DNA bound FokI endonuclease. The image shows formation of the FokI dimers with recognition sites in parallel (left) and anti-parallel (right) orientation. RD – recognition domains, CD – cleavage domains. Upon DNA binding each cleavage domain is no longer sequestered by recognition domains, and is free to swing towards the DNA and form the active dimer with other cleavage domain. (*Image from Vanamee et al. 2001 [123]*)

The modular nature of FokI nuclease suggested the possibility of creation of chimeric nucleases. It was envisioned that by fusing the FokI cleavage domain to the DNA binding domains derived from heterologous protein, new, chimeric nucleases could be created with different sequence specificities. Such chimeric nucleases would be great new tools for genome editing purposes. The first example of such chimeric enzymes was shown by Kim and Chandrasegaran [68]. By fusing the FokI cleavage domain to the *Drosophila melanogaster* Ultrabithorax homeodomain, they obtained an active hybrid enzyme exhibiting sequence-binding preference characteristic to Ultrabithorax and cleaving DNA a few nucleotides away from its binding site. The same group later created chimeric nuclease consisting of the FokI cleavage domain fused to the DNA binding domain

of Gal4, a yeast transcription factor involved in the metabolism of galactose [69]. Other chimeric nucleases quickly followed, leading to the development of Zinc Finger Nuclease and TALEN platforms allowing creation of nucleases with custom-designed recognition sequences [13, 19, 26, and 84]. A common feature of these chimeric nucleases is the use of DNA recognition domains that can form a homodimer themselves or can bind to adjacent DNA sequences – in both cases facilitating dimerisation of FokI cleavage domains.

In the system proposed here, a protamine directs the fusion protein to the nucleus and serves as a DNA targeting domain for the FokI cleavage domain. Because protamine binds to the DNA in a sequence-nonspecific manner at many locations, and FokI cleavage domains have to form a dimer to become active, the proposed design may be able to confer non-linear dose-response characteristic of fusion nuclease discussed earlier.

1.8. Aim of the project

This project aims to design and implement a conditional genetic sterility system of pest insects, utilising DNA-targeted endonucleases, controlled by tetracycline-repressible expression system. Building of this system involves:

- testing different sperm specific promoters for this system;
- testing chosen endonucleases;
- testing specificity and conditionality of the complete system;
- testing general fitness and mating capabilities of males expressing the sperm-sterility system;

When built and tested, a complete system can be combined with additional modules, providing, for example, female lethality (RIDL), which would enable genetic sexing, and male sterility in a single strain.

Chapter 2 – Materials and Methods

2.1. DNA constructs

All plasmids were constructed using standard molecular biology techniques. The cloning of PCR fragments into pJET plasmids for sequencing was done using CloneJET PCR cloning Kit from Fermentas, according to the manufacturer's instructions. Only the constructs that were made by the researcher are listed below.

2.1.1. OX4280 plasmid

(Contains fusion of progesterone receptor ligand binding domain with protamine and FokI cleavage domain driven by *Ceratitis capitata* β2-tubulin promoter)

A protamine-FokI nuclease fragment was amplified from OX4089 plasmid with the following primers:

APnucleasF (ACTTCCAGAGCACTAGTATGGCCAACGGATGTCCAC) and

APnucleaSgrr (ATGATCAGTTCACCGGTGCTATTAGAAGTTGATCTCGCCGTTGTTG)

producing a 965 bp fragment.

Human progesterone receptor ligand binding domain (hPR-LBD) (with Tobacco Etch Virus protease cleavage site) was amplified from custom designed plasmid from GeneArt using primers:

hRPNhe (GAAGTTACCGCTAGCGCCACCATGGGCAAGAAGTTCAACAA) and

hRPjoinR (GGCCATACTAGTGCTCTGGAAGTACAGGTTCTC)

giving an 891 bp fragment.

Both the hPR-LBD and Protamine-FokI fragments were merged by fusion PCR [66] with the hRPNhe and APnucleaSgr primers, cloned into pJET and sequenced. The NheI-SgrAI fragment from pJET, with both PCR products, was ligated with a 10767 bp NheI-SgrAI fragment from the OX4178 plasmid bringing the hPR-LBD-TEVsite-Protamine-FokI fusion under the control of medfly β2-tubulin promoter.

2.1.2. OX4281 plasmid

(Contains fusion of progesterone receptor ligand binding domain with protamine and FokI cleavage domain driven by *Ceratitis capitata* β2-tubulin promoter; and TEV protease cds under the control of the tetO operator and the hsp70 minimal promoter)

A SpeI-NheI fragment from a custom made plasmid from GeneArt containing Tobacco Etch Virus (TEV) protease was subcloned into a NheI-SgrAI digested OX3368 plasmid placing TEV protease in front of the tetO operator and hsp70 minimal promoter. An AscI-AflII fragment from the resulting plasmid, containing the tetO-hsp70-TEV protease, was further subcloned into an AscI-AflII cut OX3079 plasmid, replacing tTAV, and creating OX4257. To create the final construct OX4280 was sequentially cut with AscI and KpnI – to allow blunting of the AscI overhang – and ligated to OX2514 bp SmaI-KpnI fragment from OX4257 containing the tetO-hsp70-TEV fragment.

2.1.3. OX4445 plasmid

(Site-specific integration plasmid containing protamine-turboGFP-EcoRI cds fusion which is under the control of the *Aedes aegypti* protamine promoter)

A protamine-turbo GFP fragment from the OX3879 plasmid was amplified with primers:

Aeprot-EcoRI-F (CAACAAGAATTCGCCGTCAGCGTCGTAGACCAAGCCG)

SG4-tGFP-Bpu10I-R (CCACCGCCTGAGCCACCGCCTCCAGATTCTTCACCGGCATCTGC)

producing an 850 bp PCR product.

EcoRI was amplified from a custom designed plasmid from GeneArt with primers:

SG4-EcoRI-Bpu10I-F (GGAGGCGGTGGCTCAGGCGGTGGAGG)

and EcoRI-2xStop-XbaI-R (TCGGGATCTAGATTATTACTTGCTGGTCAGCTG)

producing an 876 bp product.

Both fragments were merged by fusion PCR using Aeprot-EcoRI and EcoRI-2xStop-XbaI-R primers, cloned into a pJET plasmid and sequenced. A 1689 bp EcoRI-XbaI fragment with merged PCR products was cut out from pJET and ligated with a 5655 bp EcoRI-XbaI fragment from the 3878 plasmid.

2.1.4. OX4446 plasmid

(Site-specific integration plasmid containing protamine-turboGFP-FokI cleavage domain fusion under the control of *Aedes aegypti* protamine promoter)

A protamine-turbo GFP fragment from the OX3879 plasmid was amplified with following primers:

Aeprot-EcoRI-F (CAACAAGAATTCGCCGTCAGCGTCGTAGACCAAGCCG)

SG4-tGFP-Bpu10I-R (CCACCGCCTGAGCCACCGCCTCCAGATTCTTCACCGGCATCTGC) producing an 850 bp PCR product.

The FokI-Cleavage Domain was amplified from the OX4281 plasmid with primers:

SG4-1FokI-Bpu10I-F (GGCGGTGGCTCAGGCGGTGGAGGCCAGCTGGTGAAGAGCGAG)

And 2FokI-Stop-XbaI-R (CGGGATCTAGATTAGAAGTTGATCTCGC)

giving a 626 bp PCR product.

Both fragments were merged by fusion PCR using Aeprot-EcoRI-F and 2FokI-Stop-XbaI-R primers, cloned into a pJET plasmid and sequenced. The EcoRI-Bpu10I GFP fragment and Bpu10I-XbaI FokI fragment from merged PCR products were ligated with a 5655 bp EcoRI-XbaI fragment from the OX3879 plasmid.

2.1.5. OX4447 plasmid

(Site-specific integration plasmid containing fusion of turboGFP-labelled protamine with progesterone receptor ligand binding domain under the control of the *Aedes aegypti* protamine promoter)

A protamine-turbo GFP fragment from the OX3879 plasmid was amplified with primers:

Aeprot-EcoRI-F (CAACAAGAATTCGCCGTCAGCGTCGTAGACCAAGCCG) and

SG4-tGFP-Bpu10I-R (CCACCGCCTGAGCCACCGCCTCCAGATTCTTCACCGGCATCTGC) giving an 850 bp PCR product.

The human progesterone receptor ligand binding domain (hPR-LBD) was amplified from an OX4281 plasmid using primers:

SG4-TEV-hPRLBD-F

(GGCGGTGGCTCAGGCGGTGGAGGCGAGAACCTGTACTTCCAGAGCATGGGCAAGAAGT TCAACAAGGTCC)

and hPRLBD-Stop-XbaI-R

(TCGGGATCTAGATTAGGTGCTGCCGGCGATCACCTCGCTCATCATCTCG)

giving an 896 bp PCR product.

Both fragments were merged by fusion PCR using Aeprot-EcoRI-F and hPRLBD-Stop-XbaI-R primers, cloned into a pJET plasmid and sequenced. The 1712 bp EcoRI-XbaI fragment with merged PCR products was ligated with 5655 bp EcoRI-XbaI fragment from OX3879 plasmid. (*This construct was designed to test cytoplasmic sequestration in the mosquito model but this part of the project was not included in the final thesis, as it was discontinued and not completely followed through. This construct, however, served as the source of some elements used in other plasmids.)*

2.1.6. OX4466 plasmid

(Contains fusion of protamine-turboGFP-EcoRI cds under the control of the *Aedes aegypti* protamine promoter in the piggyBac **transposon backbone)**

A 2919 bp PacI-NotI fragment containing the Prot-tGFP-EcoRI fusion with protamine 5' and 3' regulatory sequences was cut out from the OX4445 plasmid. The digest was sequential with PacI digest done first to allow blunting of the produced overhang. The 7941 bp AflII-NotI fragment from the OX4184 plasmid containing piggyBac ends, ampicillin resistance gene and hr5IE1 driven DsRed was also sequentially digested to allow blunting of the AflII produced end. Both fragments were then ligated.

2.1.7. OX4467 plasmid

(Contains protamine-turboGFP-FokI cleavage domain fusion under the control of the *Aedes aegypti* protamine promoter in the piggyBac **transposon backbone)**

Done in the same way as the 4466 but PacI-NotI fragment derived from the OX4446 plasmid.

2.1.8. OX4468 plasmid

(Contains fusion of turboGFP-labelled protamine with progesterone receptor ligand binding domain under the control of the *Aedes aegypti* protamine promoter in piggyBac **transposon backbone)**

Done in the same way as the OX4466 but PacI-NotI fragment derived from the OX4447 plasmid. (*This construct was designed to test cytoplasmic sequestration in the mosquito model but this part of the project was not included in the final thesis, as it was discontinued and not completely followed through. This construct, however, served as the source of some elements used in other plasmids.)*

2.1.9. OX4626 plasmid

(Contains EcoRI cds, split with *Drosophila melanogaster* alcohol dehydrogenase intron, under the control of the tetO operator and hsp70 minimal promoter)

To build OX4626 plasmid, 2 fragments were first obtained by PCR amplifications:

1. 373 bp element containing *Drosophila melanogaster* alcohol dehydrogenase intron 3 with portions of flanking exons (adh), followed by ubiquitin coding

sequence (ubi). This element was custom synthesised by GeneArt and delivered on the plasmid from which it was amplified with primers:

AsiSI-adh-ubi-F (GGGGCGATCGCCACCATGACCAGC) and

Adh-ubi-NruI-R (CTTCTTGGGTGGGGTGGGTCGCGAACCACCGCGCAGG)

2. 899 bp fragment containing EcoRI coding sequence, amplified from OX4466 plasmid using primers:

NruI-nls-EcoRI-F

(GTTCGCGACCCACCCCACCCAAGAAGAAGCGCAAAAGCAACAAAAAACAGAGCAACCGCC)

and EcoRI-nls-R2

(GTCCTCCACCTTCCGCTTTTTCTTGGGTCGAGATCTCTTGCTGGTCAGCTGTTCGAACAG)

These two fragments were then joined by fusion PCR using the forward primer of fragment 1 and reverse primer of fragment 2. Fused 1246 bp fragment (containing adh-ubi-EcoRI) was cloned into the pJET plasmid, creating OX4623 plasmid, and sequenced. In the next step, 6593 bp AfeI-AsiSI fragment from OX3978 plasmid, 1245 bp AsiSI-SpeI fragment of OX4623, and EcoRI-nls-sv40-linker, made of two annealed oligonucleotides (5'-CTAGTCGGACCGCACGTGCCTAGGAGC-3' and 3'-TCGGACCGCACGTGCCTAGGAGC-5') were ligated together, forming OX4625 plasmid. In the last cloning step, 4574 bp NotI-BamHI fragment from OX4625 plasmid was ligated with 5981 bp fragment of OX4391plasmid. *(This construct is not discussed in this thesis; it served, however, as the source of some elements used in other plasmids.)*

2.1.10. OX4627 plasmid

(Contains fusion of protamine and FokI cleavage domain, split with *Drosophila melanogaster* alcohol dehydrogenase intron, under the control of the tetO operator and hsp70 minimal promoter)

A 359 bp fragment containing *Aedes aegypti* protamine coding sequence was amplified from OX4467 plasmid using following primers:

Aeprot-NruI-F (GATTCGCGAGCCAACGGATGTCCACCTCGACG)

and Aeprot-SG4-Bpu10I-R (CCACCGCCTGAGCCACCGCCTCCAGAGCATCGTCGTCG).

A 624 bp fragment comprising FokI nuclease cleavage domain was amplified also from OX4467 plasmid using primers:

SG4-1FokI-Bpu10I-F (GGCGGTGGCTCAGGCGGTGGAGGCCAGCTGGTGAAGAGCGAG) and FokICD-Stop-SpeI-R (ATACTAGTTTAGAAGTTGATCTCGCCGTTG).

Obtained PCR products were joined together by fusion PCR using the forward primer of the first fragment and reverse primer of the second (FokI) fragment. Resulting 963 bp PCR products was then digested with NruI and SpeI restriction enzymes and ligated with 9654 bp NruI-SpeI fragment of OX4626 plasmid.

2.1.11. OX4628 plasmid

(Contains DsRed-ZsGreen fusion, split with female-specific intron from olive fly transformer gene, under the control of the Mexican fruit fly muscle actin promoter)

To build OX4628 plasmid 4 different subparts were first obtained by PCR amplifications:

1. 2981 bp element containing the muscle actin promoter from the Mexican fruit fly fused to DsRed cds, amplified from OX4014 plasmid with primers:

SfiI-AvrII-Mex-mAct-F1

(GCATCCGGCCCGGGCGGCCCCTAGGGACCCTTGTGCCCCCTGC)

and BsmBI-sg4-DsRed-R1

(TGCGCGTCTCACTCCAGACAGGAACAGGTGGTGGCG)

2. 1215 bp fragment containing female specific intron from olive fly transformer gene, amplified from olive fly genomic DNA with primers:

BsmBI-sg4-BoTra-int-F1

(AGCGCGTCTCAGGAGGCGGAGGTAATTTTAATTGCTTACTAAATCTAGTGA)

and BsmBI-sg4-BoTra-int-R1

(TGCGCGTCTCTACCGCCGGAACCTGTGAACACGATTAATGCCAA)

3. 727 bp fragment with ZsGreen cds from OX4014, amplified using primers:

BsmBI-sg4-ZsGreen-F1 (AGCGCGTCTCACGGTGGAGGCGCCCAGTCCAAGCACGGCCT)

and BsmBI-sg4-ZsGreen-R1 (GCGACGTCTCATATCAGGGCAAGGCGGAG)

4. 691 bp fragment with Mexican fruit fly muscle actin 3'-UTR from pJET plasmid (from Dr Sarah Scaife) into which this 3'-UTR was cloned from Mexican fruit fly gDNA, amplified with primers:

BsmBI-Mex-mAct-3UTR-F1

(AGCGCGTCTCAGATAGGTCTTTTGATTGTGAAAGATGGTGA)

and BsmBI-Mex-mCt-3UTR-R2

(ACGCGCGGCCGCCGCTTAAGTTTACTTTTAGTATTTTTGCCTTGGTTAG)

All obtained subparts were cloned into pJET plasmids and sequenced. Next 2946 bp AvrII-BsmBI fragment containing mActin-DsRed, 1189 bp BsmBI fragment with olive fly transformer intron, 701 bp BsmBI fragment with ZsGreen cds, and 670 bp

BsmBI-NotI fragment containing mActin 3'-UTR were ligated with 5274 bp, NotI-AvrII digested part of OX4014 plasmid.

2.1.12. OX4676 plasmid

(Contains DsRed-ZsGreen fusion, split with female-specific intron from olive fly transformer gene and with ubiquitin cds, under the control of the Mexican fruit fly muscle actin promoter)

OX4676 was constructed in the same way as OX4628 but incorporating ubiquitin fragment into the design which forces the modification of the fragment with ZsGreen coding sequence, to accommodate ubiquitin in the cloning scheme. Ubiquitin was amplified from OX4627 plasmid using primers:

BsmBI-ubi-F1 (AGCGCGTCTCACGGTGGAGGCCAGATCTTCGTCAAGACCCTGACC) and BsmBI-ubi-R1 (TGCGCGTCTCTTTGGGTGGGGTGGGACCACCGCGCAGGCGCAG) Modified ZsGreen cds containing fragment was amplified again from OX4014 plasmid with primers:

BsmBI-nls part-ZsGreen-F1

(AGCGCGTCTCACCAAGAAGAAGCGCAAAGCCCAGTCCAAGCACGGCC)

and the same reverse primer as before.

Both parts were cloned into pJET plasmids and sequenced. 245 bp BsmBI ubiquitin element and 708 bp BsmBI ZsGreen element were used in subsequent cloning.

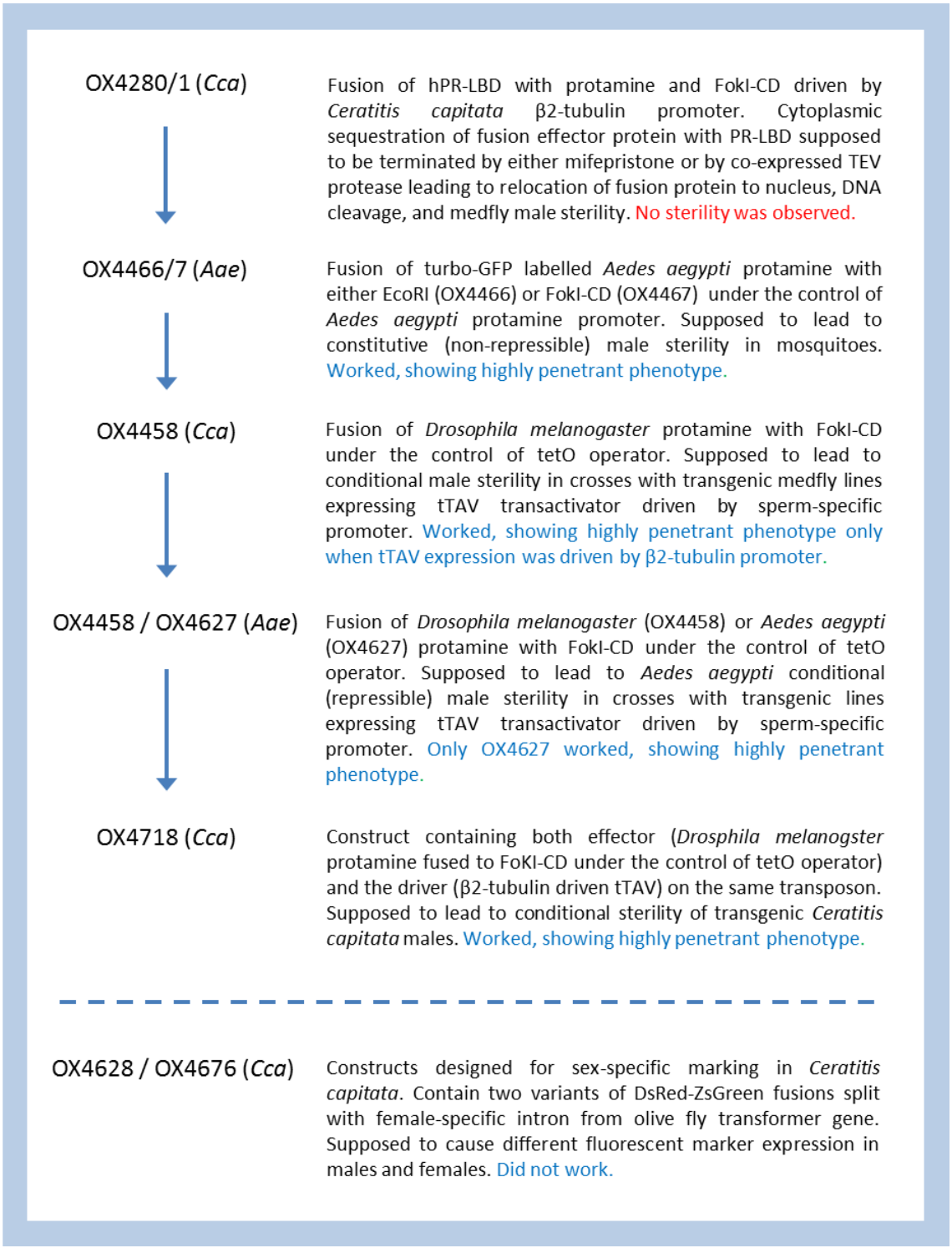


Figure 2.1 P**rogression of development of main constructs used during the project.** Upper panel contains constructs designed to induce male sterility in transgenics insects carrying them. Blue arrows indicate succession of constructs. Lower panel contains constructs designed for independent part of the project - development of sex-specific marker system. In brackets are the names of the species for which each construct was intended – *Cca: Ceratitis capitata; Aae: Aedes aegypti*.

2.2. Genomic DNA extractions

Genomic DNA extractions were performed using "GeneJet Genomic DNA Purification Kit" from Fermentas (# K0722):

1. Insect material – insect bodies with heads and wings removed – was placed in a microfuge tube. 180 µl Digestion solution was added and the sample was broken up with a sterile pestle.

2. 20 µl of Proteinase K solution was added to the microfuge tubes; samples were vortexed and incubated at 56^0C for 1-3 hours or overnight.

3. If RNA-free DNA samples were required, 20 µl RNase A solution was added, tubes vortexed and incubated for an additional 10 min at room temperature.

4. 200 µl Lysis solution was added, samples mixed thoroughly by vortexing for 15 s until a homogenous mixture was obtained.

5. Samples were centrifuged for 5 minutes at high speed to pellet insoluble particles and supernatant mixed in the clean microfuge tube with 400 µl 50% ethanol.

6. A Nucleospin tissue columns were placed into 2 ml collecting tubes; samples were applied to the columns and centrifuged for 1 minute at 6000 g (9000 rpm in the Accuspin microfuge). Flow through and collection tubes were discarded and spin columns placed in fresh collection tubes.

7. 500 µl of Wash Buffer 1 (with ethanol added) was loaded onto spin columns; samples were centrifuged for 1 minute at 8000 g (10000 rpm in the Accuspin), flow through discarded and columns placed back collecting tubes.

8. 500 µl of Wash buffer 2 (with ethanol added) was added, columns centrifuged for 3 minutes at maximum speed. The flow through was discarded and columns

placed back in collecting tubes. Columns were centrifuged for 1 minute at maximum speed.

9. Nucleospin tissue columns were put into a 1.5 ml microcentrifuge tubes and the required volume (up to 100 µl) of elution buffer was added. Samples were incubated at room temperature for 2 minutes, and then centrifuged for 1 minute at 8000 g (10000 rpm in the Accuspin) to collect eluted purified gDNA solution.

2.3. Mediterranean fruit fly methods

2.3.1. Injections

Fresh <1 hour old embryos were collected for injections and had their chorion removed by bleaching using sodium hypochlorite solution from SIGMA (10% solution of the stock). De-chorionated embryos were transferred onto a cover slip which had been treated with a thin strip of glue to hold each embryo in place. The glue was obtained by soaking double sided sticky tape in heptane. A streak of heptane extracted glue was put on each cover split and left to dry before transfer of embryos. The lining up of the embryos on the cover slips was done under a Zeiss Stemi 1000 stereomicroscope using a cold light source. Embryos were lined up so that their posterior poles were facing in the same direction. Vector/helper mix, at concentrations of 600 ng/µl and 300 ng/µl respectively, and buffered with injection buffer made up of 5 mM KCl and 1mM NaH_2PO_4 at pH 6.8, was injected into the posterior pole of the embryo. Injections were done using Eppendorf FemtoJet Express System mounted on Zeiss Axiovert 25 inverted microscope with Eppendorf Femtotips-II needles. Post injection embryos were placed on agar plates

containing apple juice until emergence. After emergence larvae were collected and placed into plates containing Mexican fruit fly diet.

2.3.2. Obtaining and rearing the transgenic lines

Surviving G_0 adult flies were sexed and crossed to wild type – separately males and females. Females are distinguished by their protruding ovipositors and different antennae – male antennae are bulb-shaped. G_1 progeny were screened for expression of transformation marker and transgenic individuals out crossed to wild type – using single transgenic fly per cross – to establish separate lines. Next generations were checked for single, autosomal transgene insertion by screening for fluorescent transformation marker. Screening was undertaken using dissection microscopes (either an Olympus SZX12 or a Leica MZFLIII) mounted with a fluorescence unit containing filters specific for the emission spectrum of each fluorescent protein.

All life stages of medfly were kept at a temperature of 26±1ºC, 65±5% relative humidity (RH) and a photoperiod of 12:12 (L:D). Larvae were fed on a *Drosophila* diet which is agar based containing maize flour, dextrose and yeast. Adults were fed with 1:4 ratio of yeast powder to sucrose. Water was supplied to adults through a wet cotton rolls. Adult medfly laid embryos through a fine mesh, which is on one side of each rearing cage. Embryos drop from this mesh into a pot of water where they can be filtered through paper and placed into a bottle with *Drosophila* diet. Once larvae begin to emerge from the food, the bottle is placed into a modified Tupperware box containing sand where they crawl out and pupate. When required, tetracycline was supplied in both larval food and in water (for adult insects) at the working concentration of 100 ng/µl. *Figure 2.2* shows the

Ceratitis capitata rearing cage, food bottles into which collected filtered eggs are placed, and plastic Tupperware box filled with sand for larvae to pupate in.

Figure 2.2 Equipment for rearing Mediterranean fruit flies. The *Ceratitis capitata* rearing cage and bottle with larval food (inside visible folded filter paper with filtered eggs) (A); plastic box

2.3.3. Egg hatch-rate assays

To assess the extent of sperm-sterility in experimental transgenic males, hatch-rate assays were performed. Details of the experimental set-ups are explained elsewhere in the thesis, when particular experiments are discussed, here, only the method for estimating hatching rate of collected eggs is presented.

Egg collections for the assay started usually around the fifth day after setting up of cages. This was to ensure that all females reached sexual maturity, mated with provided males, and had enough time after matings to commence the production of the eggs. 24 hours before intended 1st collection, all the eggs laid into the collection pots were discarded and pots supplied with clean water. This made sure that the eggs collected for the actual assay were more developmentally

synchronized, and that no hatched larvae were present among the eggs. On the day of collection of the eggs for the assay, filter papers were prepared by drawing on them a 1 cm x 1 cm grid to facilitate egg counting. Laid eggs were then collected with plastic Pasteur pipettes from collection pots (swirling the water in the pots helps to gather the eggs in the centre of the pot) and transferred onto filter papers placed in Petri dish covers with enough water to keep the eggs sufficiently moist. Eggs were then counted under the Zeiss Stemi 1000 stereoscope equipped with cold light source. Next, covered Petri dishes (with bottoms used as lids) were placed in plastic Tupperware boxes laid with wet tissue paper to sustain the moisture. 3-4 days later eggs were counted again recording numbers of not hatched eggs. Numbers of hatched eggs were then calculated as difference between collected and not hatched eggs. In the case of samples where hatching was significantly decreased, with just a few eggs hatching; numbers of hatched eggs were recorded directly – to avoid obtaining negative numbers in case of counting errors resulting in larger number of not hatched eggs then collected. A picture of filter paper with collected eggs is shown in *Figure 2.3*.

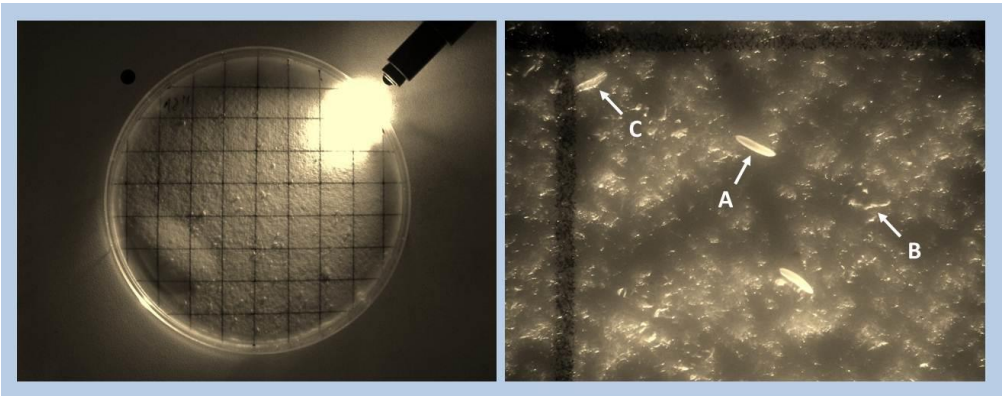


Figure 2.3 Egg counting for the hatch-rate assay. The filter paper with collected *Ceratitis capitata* eggs visible as white dots – left panel. Magnification showing not-hatched egg (A), hatched egg (B) and 1st instar larva (C) – right panel.

2.3.4. Statistical analysis of egg hatching

In cases when more than one egg collection were done during the hatch-rate assays, mean hatching rate was calculated based on hatching rates from individual collections. To assess the spread of values between separate collections, standard deviation was calculated. The Chi-square test was used to assess significance of decrease in hatching rate in experimental samples, with "on-tet" and wild type used as reference.

2.3.5. Fluorescence microscopy of testis and sperm samples

For whole testis fluorescence microscopy, testis were dissected in Phosphate Buffered Saline (PBS), placed on the slide and imaged using a Zeiss Axioskop II microscope. For sperm imaging, testes were squashed under the cover slip releasing sperm and imaged using the same microscope.

2.4. *Aedes aegypti* methods

2.4.1. Injections

Pre-blastoderm embryos were transformed by microinjection using standard protocol [59]. PiggyBac transposase was provided as mRNA generated with the MEGAscript T7 Kit (Ambion) at a concentration of 7 µg/µl diluted in nuclease free water. Embryos were injected with mRNA and 3 µg/µl of the piggyBac vector. The injection mix was supplied with injection buffer, which is made up of 5 mM KCl and

1 mM NaH_2PO_4 at pH 6.8. Injected embryos were kept for 4 days in moist environment, and then hatched in a vacuum desiccator.

2.4.2. Obtaining and rearing the transgenic lines

The methodology of obtaining transgenic lines was similar to one used in case of Mediterranean fruit fly lines. In case of male-specific dominant sterility lines were maintained through female to wild type male crosses. Males were separated from females at the pupa stage, distinguished from each other based on the end of the pupal abdominal segments below paddles, as shown in *Figure 2.4*. Female pupae are also usually larger then male pupae.

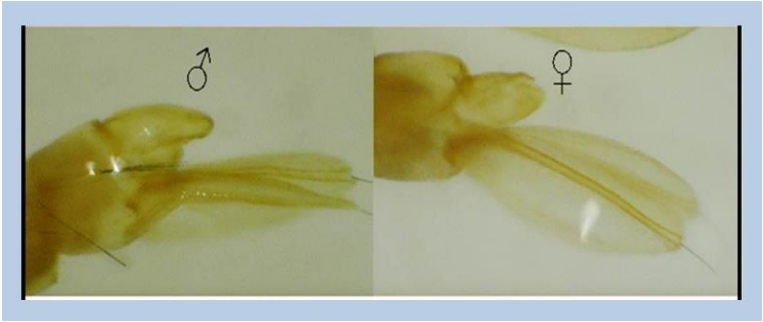


Figure 2.4 Anatomical differences between *Aedes aegypti* male and female pupae. Males (right) and females (left) can be distinguished from each other due to different shapes of ends of abdominal segments.

Mosquito larvae were fed Tetramine fish flakes, sprinkled into the water pots (or trays) containing larvae; adults were maintained on 10% sucrose solution, supplied from feeders made of plastic vials with drilled caps with cotton roll inserts. *Figure 2.5* shows the cage with adult *Aedes aegypti* mosquitoes with plastic feeder visible attached inside.

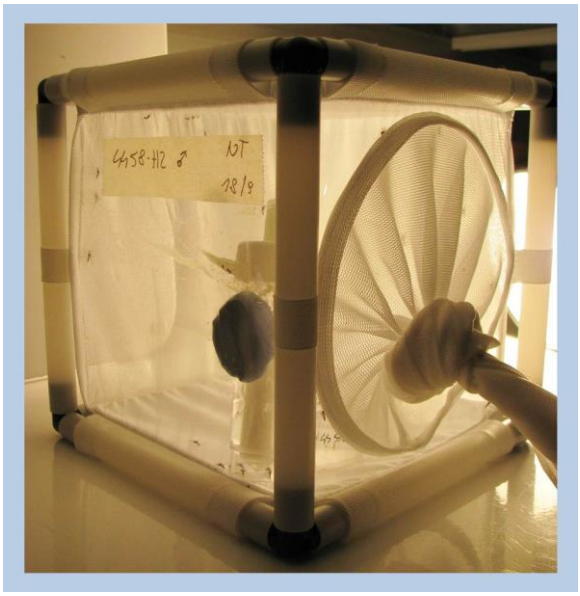


Figure 2.5 The adult *Aedes aegypti* rearing cage. Sugar water feeder visible attached to the side of the cage with Blu-Tac.

When required, tetracycline was supplied both to larvae (added to the water pots with larvae) and to adults (supplied together with sugar water) at the working concentration of 30 ng/µl. After mating, females were fed defibrinated horse blood from TCS Biosciences using specially designed feeders and at least 2 days after blood meal were allowed to lay eggs onto moist filter papers placed on a layer of wet cotton wool in the Petri dishes put into the cages. Egg hatching was induced in a desiccator.

2.4.3. Egg hatch-rate assays

The methodology of egg hatch-rate assays in *Aedes aegypti* was similar to one used in case of Mediterranean fruit flies. One difference was that eggs were laid directly onto the moist filter paper put inside the cages. Secondly, 4-5 days after collection filter papers with collected eggs were placed into plastic pots filled with water and egg hatching induced in the desiccator. After induction, 3-4 days were allowed for

completion of hatching. Filter papers were then removed, briefly dried and numbers of hatched eggs recorded. Hatched eggs were easily recognisable; with about ¼ part of egg shell at one end detached, resembling opened soft boiled eggs. Since not all eggs stayed attached to the filter paper, water in the plastic pot was always inspected for remaining eggs, which were then counted. Similar statistical analysis was performed as in case of *Ceratitis capitata*.

2.4.4. Fluorescence microscopy of testis and sperm samples

For whole testis fluorescence microscopy, testis were dissected in Phosphate Buffered Saline (PBS), placed on the slide and imaged using a Zeiss Axioskop II microscope. For sperm imaging, testes were squashed under the cover slip releasing sperm and imaged using the same microscope.

Chapter 3 – A GeneSwitch based conditional male sterility in Ceratitis capitata

3.1. Introduction

Although in theory a conditional sterility system (damaging sperm DNA and rendering males sterile) could be applied to many different species it would not be equally desirable in all cases. For example in mosquitoes a late acting lethal transgene would be more beneficial due to intraspecific larval competition for resources among transgenic and wild type larvae [95]. Although the primary goal of sterile-insect control programmes is long-term population suppression, it is clearly desirable in the case of agricultural pests, like the Mediterranean fruit fly, also to minimise the short-term damage done to the fruits hence the need to eliminate developing insects as early as possible – and sperm with damaged DNA would be capable of delivering its genetic material to the egg but not capable of promoting embryo development beyond the earliest stages. Indeed, this is how the current radiation-based Sterile Insect Technique programmes work. The Mediterranean fruit fly is believed to be the most destructive agricultural pest worldwide and is the target of several current SIT programmes. The importance of an environmentally friendly control of this pest together with the relatively easy rearing and general handling of this insect, render medfly our chosen model for the sperm lethal system.

In this project, as was explained in the introduction, male germline expression of fusion protein comprising protamine and FokI nuclease cleavage domain was

proposed as a method of inducing male sterility (paternal effect lethality) in pest insects. Such protein fusion, as explained previously, due to reliance of its nuclease activity on stochastic dimerisation events, can minimise the influence of basal or leaky expression in non-target tissues or before activation (de-repression) of the system.

Yet further phenotypic specificity and conditionality of the effector can be achieved by employing combinatorial control. Such a system would be composed of two components, neither of which is active on its own; only when present together in desired cells/tissues can they exert their effect. An example could be an independently expressed pre-protein and a protease, which by proteolytic cleavage activates the pre-protein. Expression of at least one of the components needs to be regulatable to provide conditionality of the system. Because both components have to act together in the same cells to cause any effect, this system provides additional protection against leakiness of any of the promoters used and against their off-target activity (intrinsic or resulting from a position effect). So, in other words, expression in cells other than the intended ones will have no effect except in the unlikely circumstances that both components are mis-expressed in the same cells. This means that the combinatorial system may also add more flexibility in the choice of the promoters. To achieve such combinatorial control of transgene expression in our genetic sterilisation system, incorporation of elements of the GeneSwitch system was proposed.

GeneSwitch is an inducible gene expression system originally designed by Burcin *et al.* [18] for genomic studies and gene therapy. It is similar to the GAL4/UAS system widely used in *Drosophila melanogaster* and incorporates elements involved in hormonal control of gene expression.

The Gal4 / UAS system was developed by Brand and Perrimon in 1993 and proved to be invaluable tool for characterization of genes function in *Drosophila melanogaster* and other organism [17]. The system utilises the Gal4 yeast transcription factor responsible for regulation of genes involved in metabolism of galactose. The Gal4 / UAS is composed of two elements: the Gal4 transcription factor and its binding site – the Gal4 upstream activating sequence (UAS). The expression of the Gal4 is controlled by the chosen promoter or proximity to a genomic enhancer. The Ga4 transcription factor drives in turn the expression of a target gene by binding to UAS sequence located upstream of target gene cds. This bipartite nature of Gal4 / UAS system allows for rapid generation of lines with expression of target gene directed to different tissues – depending on the selected promoter. It also allows, through separation of activator and target gene into separate lines, to easily maintain lines containing otherwise lethal alleles

[17, 25, 94]

Nevertheless, the technique provides no control over the timing of GAL4 expression. This additional control has been added in the Gal4 / UAS modification, termed GeneSwitch, in the form ligand-inducible transactivation.

In the GeneSwitch system, the gene of interest, the expression of which is to be regulated, is under the control of a UAS operator. The transactivator of the gene of interest comprises the GAL4 DNA binding domain (binding to a UAS operator) fused to a human progesterone receptor ligand binding domain (hPR-LBD) and to the human NF-kB activation domain subunit – p65 [99].

After expression of GAL4-hPRLBD-p65, the ligand binding domain of this fusion protein monomer interacts with heat shock proteins forming a complex that is sequestered in the cytoplasm and therefore inert and unable to drive the

expression of the gene of interest from the UAS operator. Induction of expression is caused by administering the steroid hormone analogue, mifepristone (RU486). Mifepristone binds to the progesterone receptor ligand binding domain, inducing conformational change in the monomeric polypeptide structure leading to release of heat shock proteins. This terminates cytoplasmic sequestration and the whole fusion protein is able to translocate to the nucleus. In the nucleus monomeric fusion proteins dimerise through the action of GAL4 domains, and the resulting homo-dimers are able to bind to UAS operator and drive the expression of the gene of interest – through the recruitment of transcription initiation complex by p65 activation domain [Inovio Inc. website, 89, 99]. A simplified diagram of GeneSwitch system function is shown in *Figure 3.1*.

GeneSwitch system has been successfully used to spatially and temporarily control the transgene expression in *Drosophila melanogaster,* with the target expression detectable in 0.5 – 1h after induction with the mifepristone [89, 99].

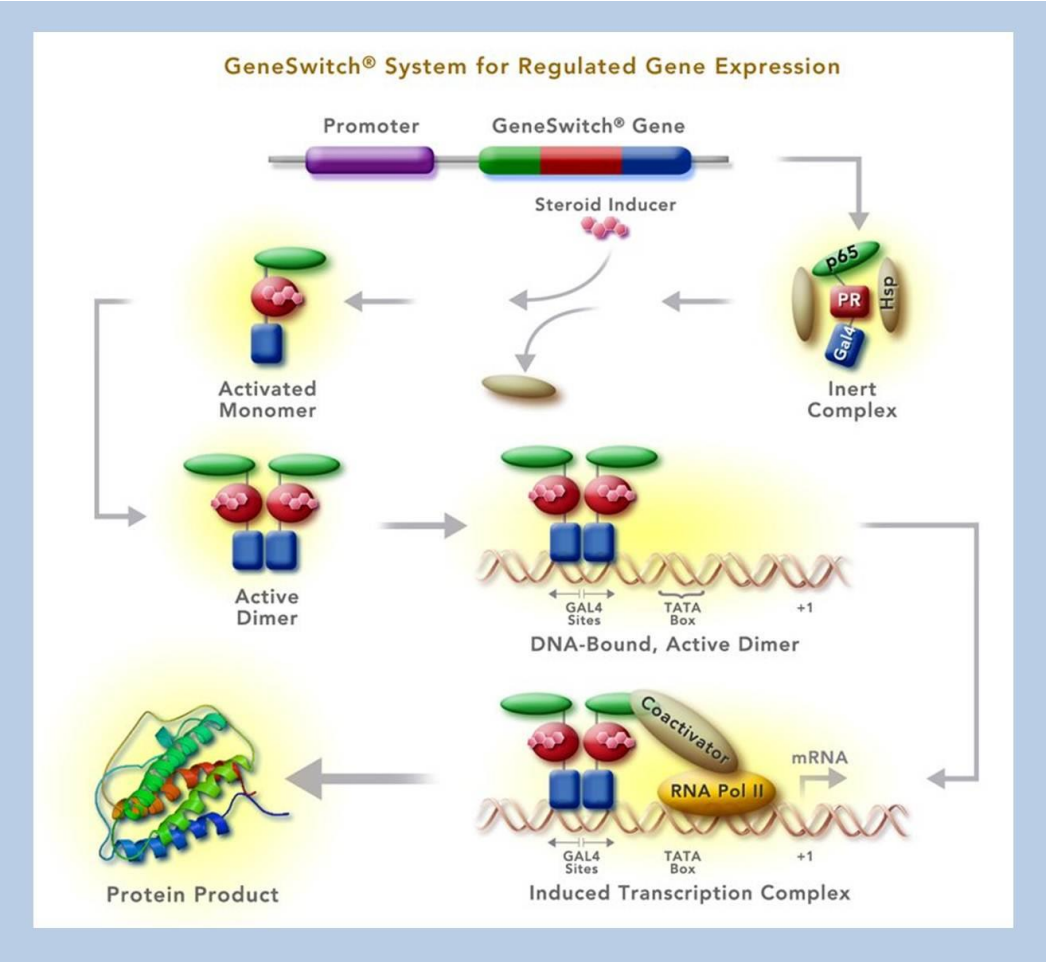


Figure 3.1 Schematic diagram of GeneSwitch system. In GeneSwitch system gene of interest is under the control of a UAS operator. The transactivator comprises the GAL4 DNA binding domain (binding to a UAS operator) fused to a human progesterone receptor ligand binding domain (hPR-LBD) and to the human NF-kB activation domain subunit – p65. Expressed GAL4-hPRLBD-HF-kB fusion protein is sequestered in the cytoplasm through interactions of hPR-LBD with heat shock proteins. Administering the mifepristone, which binds to hPR-LBD, causes conformational change in the fusion protein leading to release of heat shock proteins. GAL4-hPRLBD-p65 fusion is no longer sequestered in the cytoplasm and can translocate to the nucleus where it binds to the UAS operator inducing transcription of the gene of interest. *Image © 2002 Inovio, Inc.*

To implement combinatorial control in our genetic sterilisation system we used the sequestering properties of the progesterone receptor ligand binding domain (hPR-LBD). Our chosen nuclease – protamine-FokI cleavage domain fusion – is fused to hPR-LBD. This final fusion forms one component of the combinatorial

system, an inactive pre-protein, inactivity of which is ensured by aforementioned cytoplasmic sequestration.

Although this inert complex can be activated by administering mifepristone such a method of activation would be rather difficult to perform in the mass-rearing facility due to mifepristone toxicity and cost [89, 99].

In our proposed design, activation of the pro-toxin is achieved by removal of the ligand binding domain from the whole fusion protein. This is done by the action of the Tobacco Etch Virus protease (TEV protease). TEV protease is a cysteine protease commonly used to cleave affinity tags from recombinant proteins. TEV protease is more versatile than many other proteases due to its high specificity and activity at a wide range of physiological conditions [50, 65]. In the system proposed here, it is supposed to cleave at its recognition site introduced between the ligand binding domain and the rest of the fusion protein, removing the former. Protamine-FokI nuclease, no longer sequestered in the cytoplasm, becomes an active protein capable of entering the nucleus and targeting DNA. TEV protease thus forms the second component of combinatorial control.

Two constructs were built to test the feasibility of using GeneSwitch modification as the elements of combinatorial control for a male-germline-specific sterility system, OX4280 and OX4281.

OX4280 is single-ended piggyBac construct with DsRed under the control of the hr5IE1 promoter as a transformation marker. The 'Sperm sterility module' comprises a human progesterone receptor ligand binding domain (hPR-LBD) fused with an *Aedes aegypti* protamine (AeProt) and a single FokI cleavage domain (FokICD) under the control of a Mediterranean fruit fly β2-tubulin promoter – a 1030 bp fragment of endogenous *C. capitata* β2-promoter including part of 5' UTR.

*Drosophila melanogaster fs(1)*K10 gene derived 3'UTR was used as the 3' regulatory sequence – This 3'UTR has been used in Oxitec's constructs as an alternative to the common SV40 3'UTR. *Drosophila melanogaster fs*(1)K10 is an early acting gene, involved in establishing oocyte dorso-ventral polarity [44].

The purpose of this construct was to test the induction of sterility with mifepristone – to avoid any interference from additional genetic elements not necessary for this kind of activation; the tetO-TEV part is omitted.

The TEV protease – the second element of the combinatorial system – is incorporated in the second construct, OX4281, which is essentially OX4280 transposon with addition of the Tobacco Etch Virus (TEV) protease coding sequence under the control of a tetracycline operator (tetO), and *D. melanogaster fs(1)*K10 3'-UTR.

The hPR-LBD – Protamine – FokICD fusion in its "native" state is intended to be an inactive pro-toxin. It is designed to be activated in two ways:

1. By expression of TEV protease which cleaves off sequestered fusion-nuclease at the TEV cleavage site between the hPR-LBD and the protamine domain, releasing the protamine-FokICD fusion, which can now enter the nucleus. Protamine serves as a DNA binding domain targeting the non-specific FokI cleavage domain to DNA. TEV expression can be achieved by crossing the OX4281 line with a line expressing tTAV under the control of a suitable promoter; alternatively, in principle, a separate TEV protease line could be used.
2. By activation with RU486 (mifepristone) which binds to hPR-LBD and by causing a conformational change releases bound chaperones thereby terminating fusion-nuclease sequestration in the cytosol – the hPR-

protamine-nuclease fusion is then free to enter the nucleus and bind to DNA. In case of activation with mifepristone there is no need to cross OX4281 line to a tTAV expressing line.

OX4280 offers only the first method of activation, OX4281 offers both. Schematic structures of both constructs are shown in *Figure 3.2*.

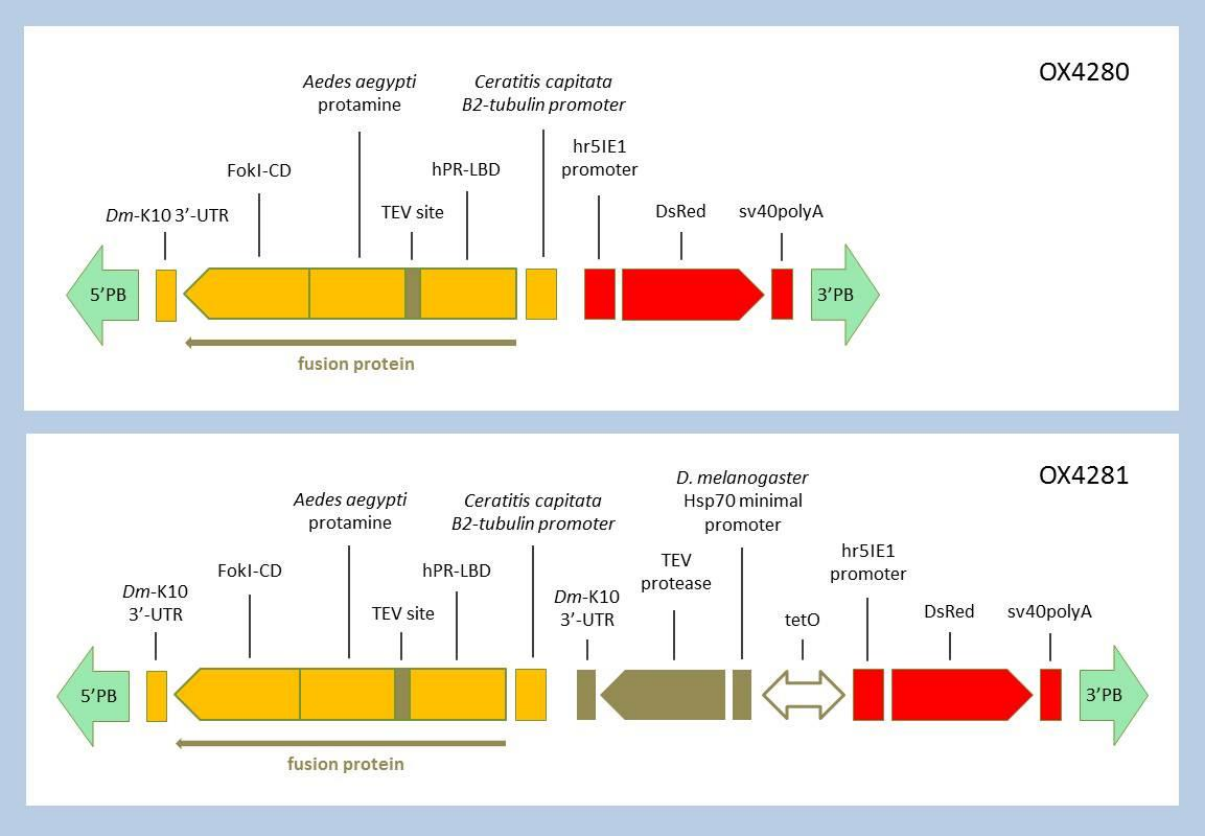


Figure 3.2 Schematic diagrams of OX4280 and OX4282 constructs. OX4280 contains the fusion of hPR-LBD with protamine and FokICD driven by *Ceratitis capitata* β2-tubulin promoter. Cytoplasmic sequestration of fusion effector protein with PR-LBD can be terminated by mifepristone leading to relocation of fusion protein to nucleus, DNA cleavage, and male sterility. OX4281 also contains hPRLBD-protamine-FokICD fusion which can be activated by administering mifepristone. It differs from OX4280 in that it additionally contains TEV protease cds under the control of tetO operator. When expressed, in the presence of tTAV transactivator, TEV protease should cleave off the hPR-LBD terminating cytoplasmic sequestration of the effector.

3.2. Results

3.2.1. Obtaining OX4280 lines

OX4280 plasmid was co-injected into pre-blastoderm medfly embryos with the OX3022 helper plasmid as the source of the piggyBac transposase. Around 1000 eggs were injected in total. Injections were performed on two consecutive days – these are later referred to as injection set 1 and 2. Post-injection survival was as follows:

> 258 larvae → 169 pupae (of which 95 showed transient expression of fluorescent marker) → 157 adults (83 males + 74 females)

All G_0 adults (both transiently expressing fluorescent marker and not) were divided into cages (separately males and females) and out crossed to wild type flies. G_1 progeny were screened for the expression of a fluorescent transformation marker (DsRed), and transgenic individuals were discovered among progeny from 12 crosses. At this stage crosses of single transgenic flies from each pool with wild type were set up to establish separate lines, each representing a different insertion event. Insects from subsequent generations were tested for similar wild type to transgenic and male to female ratios as an indication of single versus multiple insertions and autosomal versus sex chromosome insertion respectively. Phenotype segregation in obtained lines is showed in *Table 3.1*.

Two lines, OX4280-B2-I and OX4280-G1-II were ultimately chosen for continued breeding as ones with arguably the strongest phenotype (DsRed expression level) among lines with a single insertion event.

OX4280-A2-I	219 wild type	(99 ♀ + 120 ♂)	and	208 transgenic	(99 ♀ + 109 ♂)
OX4280-B2-I	184 wild type	(103 ♀ + 81 ♂)	and	163 transgenic	(70 ♀ + 93 ♂)
OX4280-A1-II	120 wild type	(47 ♀ + 73 ♂)	and	91 transgenic	(51 ♀ + 40 ♂)
OX4280-B1-II	204 wild type	(111 ♀ + 93 ♂)	and	192 transgenic	(98 ♀ + 94 ♂)
OX4280-B2-II	51 wild type	(0 ♀ + 51 ♂)	and	352 transgenic	(179 ♀ + 173 ♂)
OX4280-D1-II	146 wild type	(73 ♀ + 73 ♂)	and	136 transgenic	(63 ♀ + 73 ♂)
OX4280-D2-II	203 wild type	(102 ♀ + 101 ♂)	and	189 transgenic	(94 ♀ + 95 ♂)
OX4280-G1-II	196 wild type	(92 ♀ + 104 ♂)	and	195 transgenic	(77 ♀ + 118 ♂)

Table 3.1 Phenotype segregation in OX4280 *Ceratitis capitata* lines. Wild type to transgenic and male to female ratios in G_3 – A2-I, B2-I and G_2 – A1-II – G1-II OX4280 lines were assessed to select lines with single, autosomal insertion exhibiting strong expression of fluorescent marker. Two lines, OX4280-B2-I and OX4280-G1-II were ultimately chosen for continued breeding. Roman numerals I and II denotes lines coming from either first or second injection sets.

3.2.2. Obtaining OX4281 lines

OX4281 plasmid was co-injected into pre-blastoderm medfly embryos with OX3022 helper plasmid as the source of the piggyBac transposase. Around 1000 eggs were injected on two consecutive days (injection set 1 and injection set 2) in total. Post-injection survival was as follows:

> 343 larvae → 256 pupae (of which 178 showed transient expression of fluorescent marker) → 196 adults (91 males + 105 females)

Lines were obtained in a similar way as for OX4280 construct. Insects from subsequent generations were tested for similar wild type to transgenic, and male to female ratios as an indication of single or multiple insertions and autosomal or sex chromosome insertion respectively. Phenotype segregation in obtained lines is showed in *Table 3.2*.

Two lines, OX4281-F-II and OX4281-P-II were selected to be bred as they showed the strongest DsRed expression.

OX4281-A1-I	207 wild type	(108 ♀ + 99 ♂)	and	161 transgenic	(74 ♀ + 87 ♂)
OX4281-A1-II	219 wild type	(83 ♀ + 95 ♂)	and	208 transgenic	(120 ♀ + 129 ♂)
OX4281-E1-II	72 wild type	(0 ♀ + 72 ♂)	and	121 transgenic	(121 ♀ + 0 ♂)
OX4281-E2-II	77 wild type	(44 ♀ + 33 ♂)	and	84 transgenic	(45 ♀ + 39 ♂)
OX4281-J1-II	140 wild type	(66 ♀ + 74 ♂)	and	156 transgenic	(72 ♀ + 84 ♂)
OX4281-F-II	183 wild type	(91 ♀ + 92 ♂)	and	163 transgenic	(86 ♀ + 77 ♂)
OX4281-H-II	109 wild type	(69 ♀ + 40 ♂)	and	157 transgenic	(81 ♀ + 76 ♂)
OX4281-I-II	133 wild type	(73♀ + 60 ♂)	and	154 transgenic	(78 ♀ + 76 ♂)
OX4281-K-II	210 wild type	(104♀ + 106 ♂)	and	239 transgenic	(112 ♀ + 127 ♂)
OX4281-P-II	199 wild type	(95♀ + 104 ♂)	and	208 transgenic	(108 ♀ + 84 ♂)

Table 3.2 Phenotype segregation in OX4281 *Ceratitis capitata* lines. Wild type to transgenic and male to female ratios in G_3 – A1-I and G_2 – A1-II – P-II OX4281 lines were assessed to identify lines with single autosomal insertions. Two lines, OX4281-F-II and OX4281-P-II were kept for further experiments. Roman numerals I and II denote lines coming from either first or second injection sets.

3.2.3. Mifepristone assays

At the time when OX4281 and OX4280 constructs were produced, no suitable tested, tTAV expressing lines in *Ceratitis capitata* were available yet, and so induction with mifepristone was the only method to test the activation of pre-protein. Hence, only OX4280 lines were used to avoid issues of potential interference from tetO-TEV. Mifepristone (also known as RU486) is toxic to humans by inhalation or ingestion so to limit human exposure to it, and the amount of RU486 waste, it was decided that only one line, OX4280-B2-I, would be used in this experiment. As a further precaution all experimental cages were

contained in large, sealed plastic boxes. Four cages were set up for the experiment – the experimental set up is shown in *Table 3.3*.

	Treatment (+mifepristone)	**Control** (- mifepristone)
OX4280-B2-I	**Cage 1**: 5 ♂ x 10 Wt ♀	**Cage 2**: 5 ♂ x 10 Wt ♀
Wt	**Cage 3**: 5 ♂ x 10 Wt ♀	**Cage 4**: 5 ♂ x 10 Wt ♀

Table 3.3 Design of OX4280 mifepristone assay.

Two separate experiments were carried out. In the first experiment mifepristone was given to adults as a water solution but due to solubility issues of mifepristone some precipitation was observed which raised the question of whether the drug was actually ingested by the insects and in what quantities. Additionally, because of the need to use containment, each cage experienced increased humidity, and the dry food used in the cages – 4:1 mixtures of sucrose and yeast extract – turned into syrup. This worked like a trap attracting and killing flies within couple of days. Thus virtually no eggs were collected.

Because of these initial problems, a second experiment was conducted. This time those individuals who were in the treatment group of experimental crosses were fed mifepristone from early larval stages onwards. At the larval stages mifepristone was supplied in the larval food – which is essentially a mixture of sucrose, maize meal and yeast powder, jellified by addition of agar. Larval food was this time used also to feed adult flies as it was less likely to turn into syrup in the

humid conditions of plastic box confinement. Additionally, RU486 administered to larvae would be earlier acting and therefore possibly more potent.

Four days after setting up the cages, eggs collected in the collection pots were removed and flies were allowed to lay new eggs for 24 h. This delay in collection and removal of old eggs was to make sure that eggs used in the experiment were of similar age, without hatched larvae among them, and that all females reached sexual maturity and the maximum of their egg laying capabilities. On the next day, all the eggs that were laid within the previous 24 h were collected and placed in the Petri dishes on wet filter papers to maintain humid conditions. On the 3rd and 4th day after egg collection filter papers were inspected for egg hatching. There was no observable difference in ratios of hatched eggs between samples – only very few eggs remained not hatched in all 4 samples, with the majority of eggs producing normal looking healthy larvae. This means that transgenic and wild type males were equally fertile, both in the presence and absence of mifepristone. This indicated that the conditional sterility system was not able to cause noticeable male sterility in transgenic males, at least in such experimental conditions.

In the first experiment concentration of the mifepristone in the water provided to flies was 50 μM (21.5 μg/ml). In the second experiment mifepristone concentration was increased to 100 μM (43 μg/ml) – both in water and solid food. According to Roman *et al.* adults of *Drosophila melanogaster* can tolerate dosages as high as 215 μg/ml (500 μM) without apparent deleterious effects [99]. Research by Ostelwalder *et al.* further narrowed down the effective range to 12-120 μg/ml and found that viability of *Drosophila melanogaster* embryos was unaffected by feeding mothers doses up to 33 μg/ml of RU486 [89]. Based on these data, the 100 μM mifepristone used in second medfly assay was thought to ensure optimal

balance between solubility in water and maximally effective, yet safe, concentration.

3.3. Conclusions

The results of the mifepristone assays prompted a detailed analysis of the design of the sterility system represented by the OX4280 and OX4281 constructs in search of possible changes and improvements.

The main advantage of these GeneSwitch-like systems is the possibility to test an effector (activation of it) independently of the rest of the system. This removes potential problems with choosing promoters to drive expression of different parts of a proposed combinatorial system.

On the other hand in its current form it has several disadvantages, including:

- Lack of obvious, simple detection of protein expression:
    1. No fluorescent protein fusion or any other tag that could be detected by antibodies (though such elements could be included in future versions of these constructs).
    2. No commercially available antibodies against FokI nuclease or FokI cleavage domain alone and most likely no antibodies against the progesterone receptor ligand binding domain (usually different parts of receptor were used as immunogen, if the sequence of immunogen was revealed).
- Lack of positive control for mifepristone assay
- Testing two things at the same time: nuclease and GeneSwitch system modification.

Several potential failure points make it difficult to troubleshoot the mifepristone assay – lack of male sterility could be due to a number of reasons. For example, such a result could be caused by problems with administering mifepristone – insects not ingesting it in sufficient quantities or not ingesting it at all, or problems with mifepristone stability. A positive control for mifepristone assay would allow testing if mifepristone is indeed accumulating in target tissues. Such positive control would need to incorporate a cytoplasmic sequestration element into the system that has been shown to work in Mediterranean fruit flies. For example one possibility would be to use tTAV fused with hPR-LBD driven by hr5IE1 promoter with tetO-DsRed used as a reporter. Such design would be a close simulation of the original GeneSwitch system with Gal4-p65 and UAS replaced by tTAV and tetO elements respectively. In the absence of mifepristone no DsRed expression should be detectable as tTAV-hPRLBD fusion should be sequestered in the cytoplasm. In the presence of mifepristone, provided it penetrates required tissues, tTAV-hPRLBD fusion should be able to translocate to the nucleus and induce DsRed expression in the pattern hr5IE1 expression.

Another, complementary, control could utilise similar design but with tTAV-hPRLBD fusion driven by sperm-specific promoter. This would allow confirmation whether administered mifepristone is ultimately able to penetrate into the germline by looking for the DsRed expression in sperm heads. A simpler version of this control could constitute DsRed fused to the hPR-LBD and driven directly by a sperm-specific promoter. In such a case, DsRed would have to be equipped with the nuclear localisation signal or fused to a DNA binding domain, for example protamine.

Promoters employed in the positive control constructs would have to be tested previously and shown to work either in bipartite or direct expression systems.

Another reason for lack of sterility in mifepristone treated flies could be impaired translocation of the hPR-Protamine-FokI fusion into the nucleus or problems with dimerisation of the FokI cleavage domains, which is required for successful cleavage [15, 123]. In chimeric nucleases utilising FokI cleavage, domain cleavage is achieved by using self-dimerising DNA binding domains or by directing two proteins to neighbouring sites. It is not entirely certain if a dimerisation of the FokI cleavage domain can be indeed mediated through protamines binding to DNA.

Additionally, in the mifepristone assay discussed here, what is actually activated (released from cytoplasmic sequestration) by the ligand is hPR-protamine-FokI fusion protein not protamine-FokI fusion, which is not quite the same as the final effector in the TEV-cleaved version. Presence of the hPR domain may cause additional problems with dimerisation by adding bulk and more constraints on the fusion protein. Similarly it may impair translocation of the activated effector into the nucleus, although in the original GeneSwitch system the hPR-GAL4-VP16 fusion showed no problems in entering the nucleus.

Addition of fluorescent protein tag to the fusion nuclease would help with troubleshooting of the aspects of the system mentioned above. It would allow for confirmation of protein expression, indicating if the promoters are working as expected. It would also provide information on the localisation of the fusion nuclease, and its successful, or not, translocation into the nucleus.

These factors suggested the need to re-design the system and perhaps test the GeneSwitch modification and nuclease separately first, before combining them together in the final construct.

The desired general features of the re-designed system were considered to be:

- Use of a dimerising nuclease – the requirement for nuclease dimerisation provides some additional protection against leaky expression in repressed state of the system by a non-linear relationship between nuclease concentration and cleavage.
- A combinatorial character of the system – two separately expressed molecules (at least one of them conditional), which only together cause the desired phenotype. This would provide further protection against leaky expression of nucleases helping to maximize insects' fitness.
- Detectable protein expression – by introducing fluorescent or epitope tags; and/or easier mRNA detection by introducing introns if possible. These modifications would help with troubleshooting the system.

Some possible modifications of the system include:

- Using a complete FokI nuclease (both domains) fusion with hPR-LBD, with or without protamine (if without, then nuclear localisation signal needed) – this can be tested first as a dominant male sterile line, without the modified GeneSwitch.
- Using a different nuclease (restriction endonuclease) – like EcoRI or NgoMIV for example. The best nuclease to use would be an endonuclease which is known to work as a fluorescent protein fusion, with typical buffer and temperature requirements. Another desired feature of such a nuclease,

from an industrial applications perspective, would be freedom-to-operate with respect to patent protection.

- Using split inteins like one which encodes a split DnaE gene of *Synechocystis* sp. PCC6803 [133] to split and trans-splice two FokI domains for example. Using a split intein gives both combinatorial characteristic of the system and the advantage of using a dimerising nuclease like system as an alternative to a GeneSwitch. Such a system would additionally eliminate issues involved with use and disposal of toxic chemicals. However, the efficiency with which trans-splicing would occur in an insect cell is unclear.

Chapter 4 – Non-repressible male sterility in *Aedes aegypti.*

4.1. Introduction

After the analysis of the OX4281 and OX4280 constructs it was decided to switch to *Aedes aegypti* as a model organism for tests of nuclease-induced sperm sterility systems. The reason for that was the presence of functional promoters in that model organism. There was already an available and tested *Aedes aegypti* transgenic line (OX3879-Aae) carrying a construct designed for fluorescent marking of mosquitoes' sperm heads. It utilised an *Aedes aegypti* tGFP-tagged protamine and its regulatory sequences to express fluorescent fusion protein in a sperm-specific manner. Strong green fluorescence had been detected both in whole dissected testis and in the isolated sperm of OX3879 male mosquitoes, as shown in *Figure 4.1.*

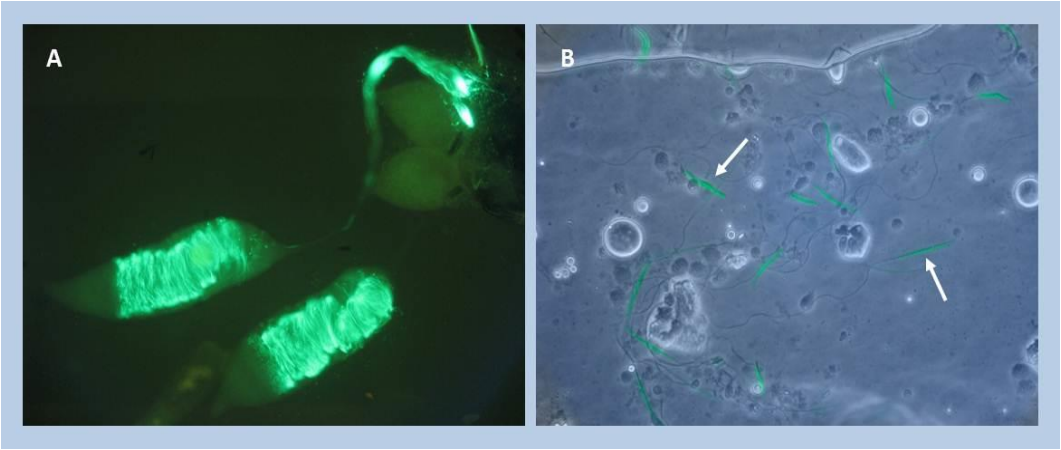


Figure 4.1 Sperm-specific expression of turboGFP in the male *Aedes aegypti* of OX3879-Aae line. Images show expression of the protamine-tGFP fusion in dissected, intact testes of OX3879-Ae male (A), and in sperm nuclei (B). Some sperm heads are marked with arrows (Images courtesy of Derric Nimmo, Oxitec Ltd.).

It was thus thought that adding another domain – a nuclease to test DNA cleavage – to the protamine-tGFP fusion was the best way to test nucleases, independently from GeneSwitch modifications, with the benefit of monitoring protein expression *via* the tGFP tag. This approach would eliminate problems associated with the bipartite system, which separates the promoter from the effector elements. Also, as protamine-tGFP fusion in OX3879 construct successfully delivered fluorescent marker into sperm nuclei, it is very likely to be able to target the FokI nuclease to sperm DNA.

Since it is not certain if protamine-FokI cleavage domain fusion would be able to induce double-strand breaks in the sperm's DNA, another nuclease – EcoRI – was also tested.

EcoRI belongs to class II restriction endonucleases and was the first restrictase discovered in *Escherichia coli*. Members of this class of nucleases typically recognise short, 4 to 8 base pairs long, usually palindromic sequences, with DNA cleavage occurring either within recognition sequence or in close proximity. They require magnesium ions as cofactors for their enzymatic activity. EcoRI is an orthodox member of the class, acting as a homodimer and recognising palindromic GAATTC site. It binds to the major groove of DNA helix, similarly to FokI endonuclease and protamines. Cleavage of double-stranded DNA by EcoRI leads to formation of so-called cohesive (or sticky) ends with 5' AATT overhang [96].

EcoRI was chosen because it appeared to be possible to attach both the N- and C-terminal tags without altering its enzymatic activity [52, 118]. EcoRI has also been shown to cleave mitochondrial and nuclear DNA and cause cell death in targeted overexpression assays in *S. cerevisiae* [7, 118]. Lastly, its use is likely not covered

by any patent – in contrast to other more recently discovered endonucleases, like FokI.

The protamine-GFP fusion expressing OX3879 plasmid was used as a template for both EcoRI and FokI encoding constructs. OX3879 is a plasmid designed for site-specific integration and this characteristic was retained in the first versions of the EcoRI and FokI constructs. However, difficulties in obtaining transgenic lines forced modifications of both plasmids. Final OX4466 (EcoRI) and OX4467 (FokI) constructs are based on single ended piggyBac transposons with hr5IE1 driven DsRed as a transformation marker and a protamine-tGFP-nuclease fusion under the control of endogenous protamine regulatory elements. Both plasmids contain constitutive expression cassettes, without an inducible or repressible on/off switch. Consequently, if these constructs achieved the intended phenotype – dominant male-specific sterility or paternal effect lethality, transgenic lines would have to be maintained through heterozygous females. The structure of the original OX3879 plasmid and its modified versions expressing nuclease fusion proteins are shown in *Figure 4.2*.

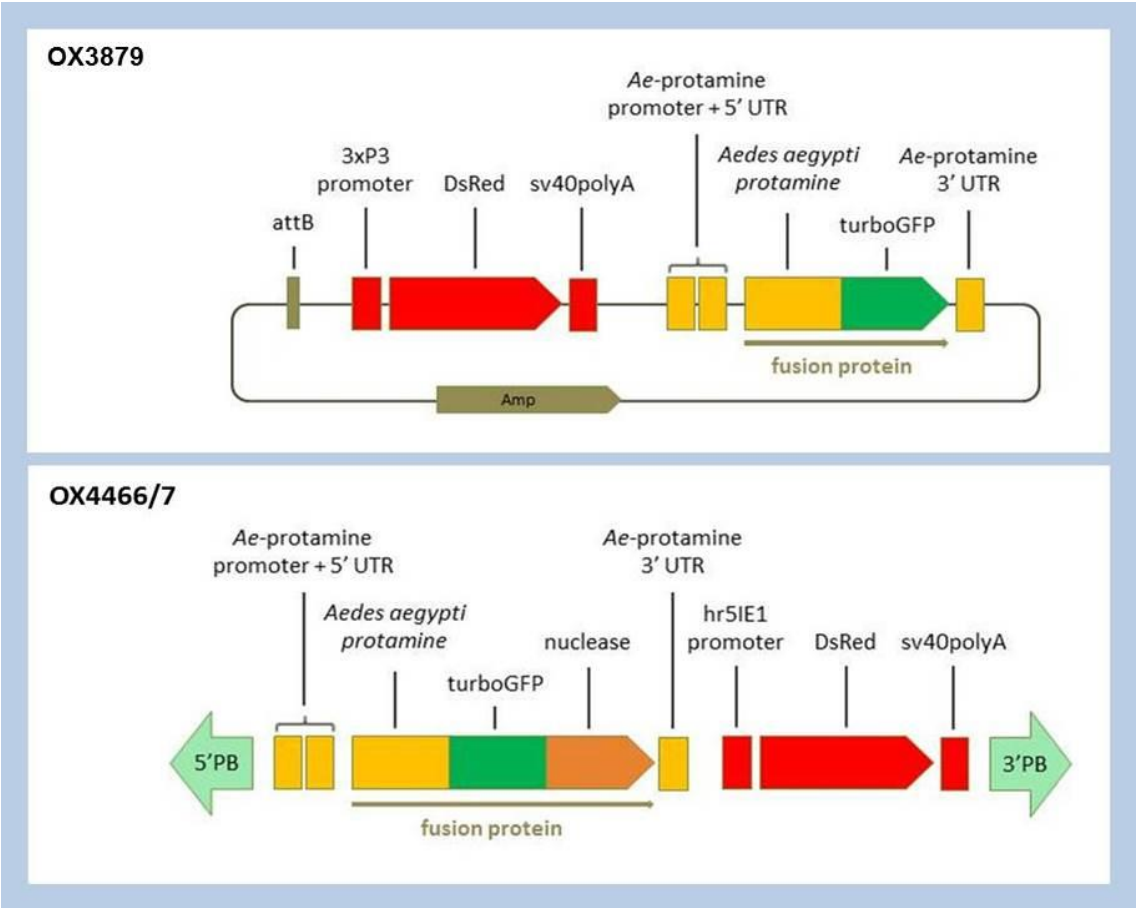


Figure 4.2 Schematic diagrams of OX3879, OX4466 and OX4467 constructs. OX3879 construct contains *Aedes aegypti* protamine fused with turboGFP under the control of protamine promoter. It was designed to test fluorescent sperm marking. Transgenic male *Aedes aegypti* mosquitoes carrying the OX3879 allele expressed tGFP in the sperm heads. OX4466/7 transposons were based on OX3879 construct. OX4466 contains the EcoRI coding sequence fused to turboGFP-labelled protamine while, OX4467 the sequence of FokI nuclease cleavage domain (FokICD). OX4466 and OX4467 are supposed to lead to constitutive (non-repressible) male sterility.

4.2. Results

4.2.1. Obtaining OX4466 lines (protamine-tGFP-EcoRI)

The OX4466 plasmid was co-injected into pre-blastoderm mosquito embryos together with transposase mRNA – as described in Materials and Methods. 1317 eggs were injected in total. Post-injection survival was as follows:

59 larvae → 55 pupae → 49 adults (23 males + 26 females)

All surviving G_0 adults – separated into males and females – were crossed to wild type - in 4 crosses:

OX4466-A: 13 ♀ x 13 wt ♂

OX4466-B: 13 ♀ x 13 wt ♂

OX4466-C: 12 ♂ x 40 wt ♀

OX4466-D: 11 ♂ x 40 wt ♀

G_1 progeny were screened for DsRed expression and transgenics were found in all 4 groups. This was not a very promising sign as it suggested that G_0 males with an integrated transposon may not have been sterile. Nevertheless G_1 crosses with wild type mosquitoes were set up using single transgenic males or females from each of 4 groups. G_2 eggs from male crosses were assayed for any changes in hatch rates [*Fig. 4.3]*. G_2 eggs from female crosses were collected from 4 (B1, B2, C1, D) out of 6 cages (A, and C1 did not produce any eggs). The B2 line was discarded as only males were transgenic. Remaining lines B1, C1 and D were kept, their analysis shown in *Table 4.1*.

	Transgenic		wild type	
	♀	♂	♀	♂
OX4466-B1	23	19	19	17
OX4466-C1	37	52	61	51
OX4466-D	46	48	59	62

Table 4.1 P**henotype segregation in OX4466 *Aedes aegypti* lines**. Wild type to transgenic and male to female ratios in G_3 OX4466 lines were used to test for presence of single autosomal insertions of OX4466 transposon.

4.2.2. OX4466 (protamine-tGFP-EcoRI) hatch rate assays

The OX4466 construct was tested using the hatch rate assay. If the OX4466 plasmid is functioning as intended, it should cause sperm DNA cleavage and render adult male mosquitoes sterile. This sterility was tested by assessing the proportion of unhatched eggs fertilised by tested males. The first hatch rate assay was performed with G_1 males – from A, B, and C G_0 cages – simply crossed to wild type females. No controls were used in this first experiment. The total number of laid eggs was counted then hatching was induced and after at least 3 days (to make sure that all the eggs were given enough time to hatch) unhatched eggs were counted again. Results of this first assay were very encouraging, showing substantially reduced hatching rate of eggs fathered by transgenic males Data from this experiment is shown below in *Figure 4.3*.

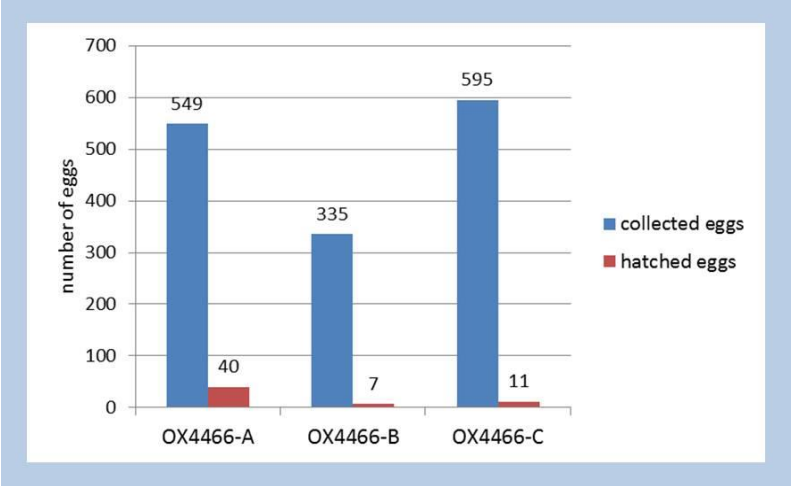
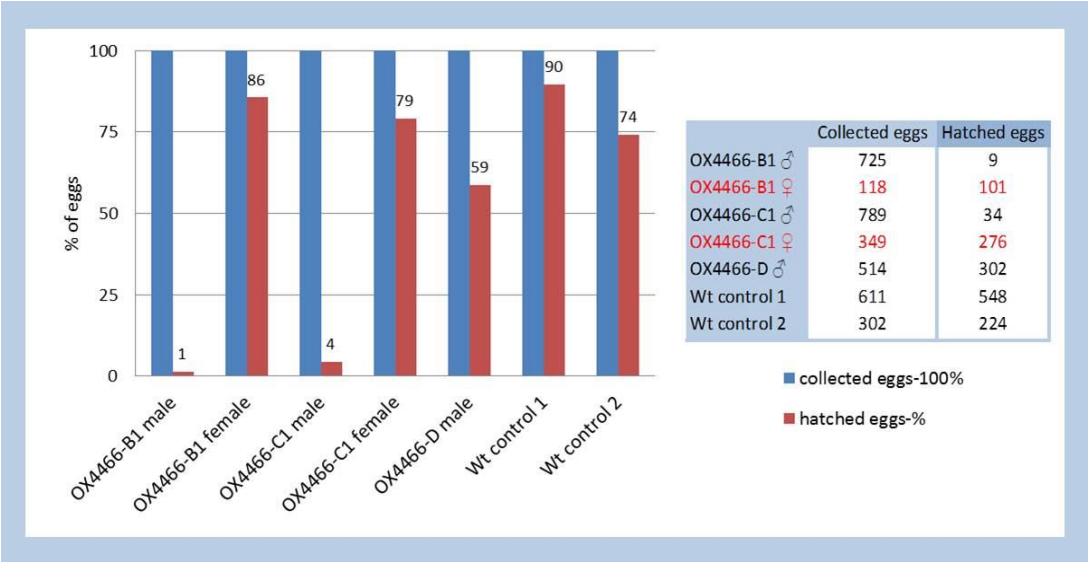


Figure 4.3 Non-repressible, constitutive male sterility in OX4466-Ae lines – 1st experiment. Transgenic males from OX4466 lines A, B and C were crosses to wild type females to test for the male sterility. Total number of eggs laid by mated wild type females was counted then hatching was induced and after at least 3 days unhatched eggs were counted again. Very few eggs hatched indicating strong male sterile phenotype.

The hatch-rate experiment was repeated with G_2 males. This time both transgenic females with wild type males' crosses and wild type only crosses were used as controls. Assessing hatch rates of eggs from transgenic females would allow us to see whether any observed effect is sex specific while the wild type control served as a reference point. Unfortunately, due to the difficulties of maintaining the lines through female crosses – using only 1 female for G_1 crosses to create truly separate lines – the original line A was lost and the assay could not be repeated for it. It was also not possible to set up a transgenic female control cross for line D. Our second experiment confirmed the rather dramatic reduction in the number of hatched eggs when these were fertilised by transgenic males – with the exception of line D. The experiment also confirmed expected sex-specificity of the sterility – eggs laid by transgenic females were hatching at almost the same proportion as wild type eggs [*Fig. 4.4*].



	Collected eggs	Hatched eggs
OX4466-B1 ♂	725	9
OX4466-B1 ♀	118	101
OX4466-C1 ♂	789	34
OX4466-C1 ♀	349	276
OX4466-D ♂	514	302
Wt control 1	611	548
Wt control 2	302	224

Figure 4.4 Non-repressible, constitutive male sterility in OX4466-Ae lines – 2nd experiment. Transgenic *Aedes aegypti* males from OX4466 B1, C1 and D lines were crossed to wild type females. Transgenic females crossed to wild type males were used as control of sex-specificity of the sterile phenotype. Wild type control was also included. Total number of laid eggs was counted for each cross and at least 3 days later numbers of unhatched eggs were recorded. The chart shows % of collected eggs that hatched from each test cross. The inside table presents actual recorded numbers. Results indicated very strong, males-specific sterile phenotype.

To make sure that the minor effect observed in line D was not an artefact – for example as a result of the presence of wild type males in the cage – B1, C1 and D line males were again crossed to wild type females. The results confirmed the differences between the lines [*Fig. 4.5*] – which are most likely due to a position effect: differential transcriptional activity of different chromosomal regions into which the transgenes have integrated. This could be supported by noticeably weaker DsRed expression in line D.

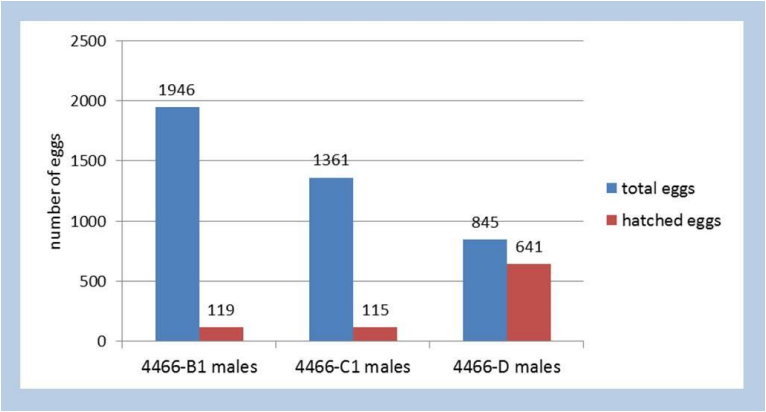


Figure 4.5 Non-repressible, constitutive male sterility in OX4466-Ae lines – 3rd experiment. Transgenic *Aedes aegypti* males from OX4466 lines B1, C1 and D were crossed to wild type females. Eggs laid by mated females were assayed for their hatchability in the similar way as in the 1st experiment – by comparing total numbers of collected eggs to the number of collected eggs that hatched upon induction. Results confirmed differences in hatch-rates between lines.

4.2.3. OX4466 (protamine-tGFP-EcoRI) protein expression analysis

In addition to the results of hatch rates experiments protein expression was also confirmed through visualisation of turboGFP tag in expressed fusion protein. Fluorescent microscopy of sperm isolated from dissected testis showed GFP co-localisation with nucleus / sperm heads (*Fig. 4.6*).

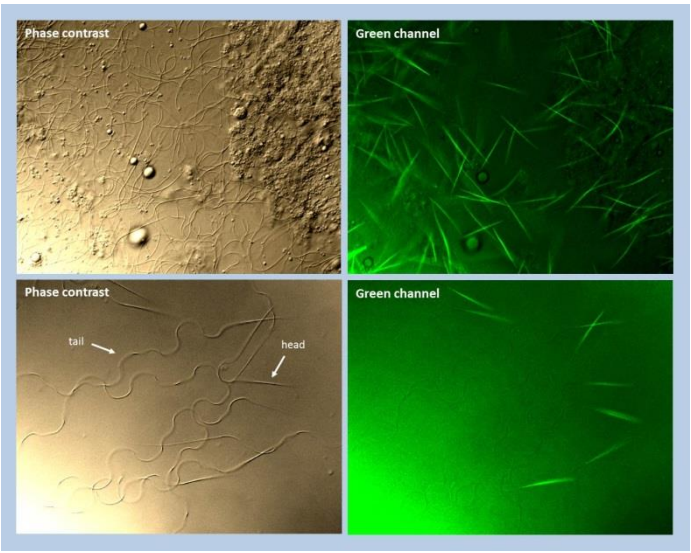


Figure 4.6 Expression of GFP-tagged fusion protein in sperm of line OX4466-B1 mosquitoes. Upper panels show area of more densely packed sperm to better visualise fluorescent sperm heads. Lower panels show area with fewer sperm to better visualise single sperms.

4.2.4. Obtaining OX4467 lines (protamine-tGFP-FokICD)

The OX4467 plasmid was co-injected into pre-blastoderm mosquito embryos together with transposase mRNA. 1545 eggs were injected in total. Post-injection survival was as follows:

213 pupae (117 males + 96 females) → 185 adults (101 males + 84 females)

11 separate G_0 crosses were set-up using all surviving adults. G_1 progeny were screened for DsRed fluorescence and transgenic individuals were found among progeny of 1 male G_0 cross (C) and 3 female G_0 crosses (E, H, I). G_1 crosses with wild type mosquitoes were set-up using single transgenic males or females from each of 4 groups. G_1 males were crossed to wild type females to check for signs of sterility. G_1 females were used to maintain the lines – two females from each line (E, H, I – line C did not produce any surviving transgenic females) in separate crosses to wild type males. Three female lines (E1, H2, and I2) produced G_2 eggs – the other females died before laying eggs. Phenotype segregation in these 3 lines is shown in *Table 4.2*. Unfortunately, only OX4467-E1 reached the G_2 stage allowing for 2nd hatch rates experiment but this was also lost in next generation (the H2 line was not used in 2nd hatch rate assay due to lack of female control).

OX4467-E1	11wild type and 6 transgenic (1 ♀ + 5 ♂)
OX4467-H2	12 wild type and 11 transgenic (1 ♀ + 10 ♂)
OX4467-I2	3 wild type and 2 transgenic (1 ♀ + 1 ♂)

Table 4.2 Phenotype segregation in OX4467 *Aedes aegypti* lines. The table shows wild type to transgenic and male to female ratios in G_2 OX4466 lines. Due to very low numbers of both pupae and adults it was difficult to test for presence of single autosomal insertions of OX4467 transposon.

4.2.5. OX4467 (protamine-tGFP-FokICD) hatch rate assays

Hatch rate experiments were conducted in a similar fashion to ones described for OX4466 lines. In the first assay only male crosses – transgenic males crossed to wild type females – were performed and no wild type control was included. Results showed a quite dramatic effect, in that no eggs hatched at all [*Fig. 4.7*].

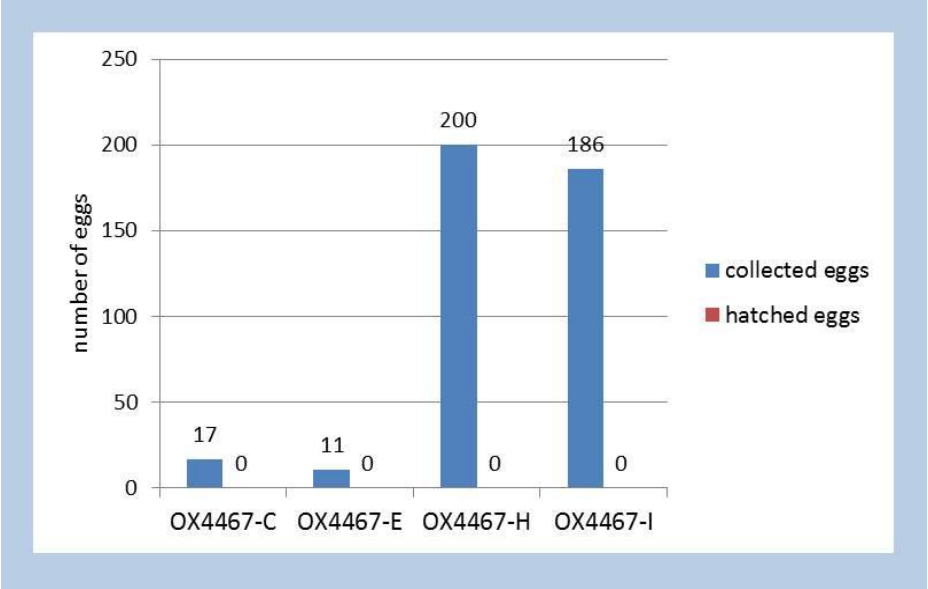
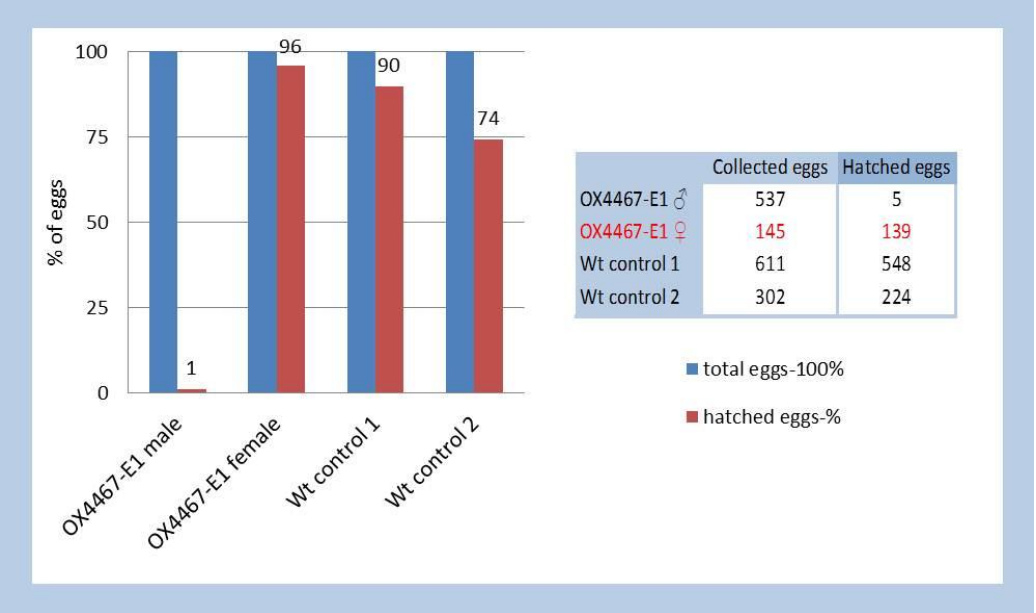


Figure 4.7 Non-repressible, constitutive male sterility in OX4467-Ae lines – 1st experiment. Transgenic males from OX4467 lines C, E, H and I were crossed to wild type females. Eggs laid by mated females were collected, counted and hatching was induced in a vacuum desiccator. Numbers of hatched eggs were recorded 4 days later. There were no hatched eggs observed in the progeny of any of the crosses indicating very strong male sterility.

The second experiment [*Fig 4.8*], this time including transgenic females crossed to wild type males as a sex specificity control, and wild type control, confirmed the preliminary results. It also showed the sex specificity of the observed effect – hatch rate of eggs obtained from transgenic females was unaffected.



	Collected eggs	Hatched eggs
OX4467-E1 ♂	537	5
OX4467-E1 ♀	145	139
Wt control 1	611	548
Wt control 2	302	224

Figure 4.8 Non-repressible, constitutive male sterility in *Aedes aegypti* OX4467-E1 line – 2nd experiment. In the second hatch-rate experiment both transgenic males and females from OX4467-E1 line were crossed to wild type *Aedes aegypti,* in separate crosses. Wild type-to-wild type control crosses were also included. Eggs obtained from each cross were counted, hatched in vacuum desiccator, and re-counted again 4 days later to record the number of hatched eggs. The chart shows % of collected eggs that hatched from each test cross. The inside table presents actual recorded numbers. The results showed very strong, male-specific sterility.

4.2.6. OX4467 (protamine-tGFP-FokICD) protein expression analysis

Protamine-tGFP-FokI fusion protein expression was additionally confirmed with fluorescence microscopy both in larvae and in single sperm cells, where it co-localised with the nucleus [*Fig. 4.9*].

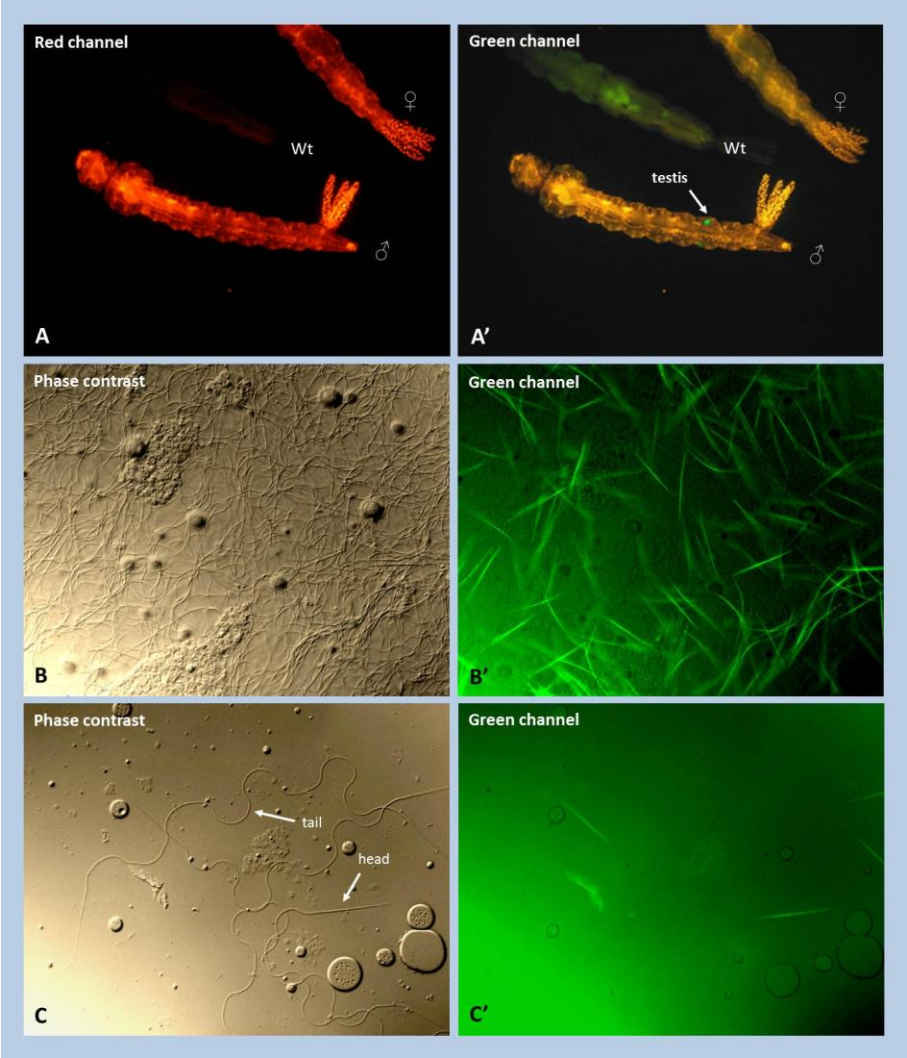


Figure 4.9 Expression of GFP-labelled protamine-FokICD fusion protein in OX4467-E1 mosquito line. A, A' – Fluorescent imaging of OX4467-E1 larvae with visible green testis; B, B', C, C' – isolated sperm with tGFP fluorescence localised in sperm nuclei. Middle panels show area of more densely packed sperm to better visualise fluorescent sperm heads. Lower panels show area with fewer sperm to better visualise single sperms.

4.3. Conclusions

Experiments in *Aedes aegypti* proved that sperm-specific expression of a nuclease can indeed cause quite high penetrance male sterility (paternal effect lethality). The experiments also confirmed the male specificity of the protamine promoter

and the DNA targeting properties of protamine itself. They also showed that both EcoRI and FokI can act as effective nucleases.

Published research suggests that EcoRI could probably be used by itself, without protamine, as it already contains a sequence specific DNA binding domain [7]. FokI, on the other hand, contains a non-specific cleavage domain and it must be targeted to DNA by a DNA binding domain like protamine – or at least, such targeting is expected to greatly increase the cleavage efficiency.

Unfortunately, the results that we have obtained do not ultimately answer the question of dimerisation of FokI in the context of a protamine-FokI fusion. Though turboGFP was originally selected in part because it was described by the manufacturer as being monomeric, more recent findings indicate that it has to self-dimerise to become active and exists as a dimer in solution [29]. It may be that the dimerisation of tGFP rather than DNA binding of protamine brings together the two FokI cleavage domains to form an active nuclease. In such a case, the protamine-tGFP-FokI fusion protein could form an active dimer even before being targeted onto DNA and would cleave DNA whenever bound to it by protamine – making it a very potent nuclease; this would also likely affect the relationship between the concentration of the fusion protein and cleavage rate. The dimerisation properties of turboGFP could actually be employed in our system if it turned out that protamine – FokICD fusion protein is not capable of forming active FokI dimers. But to conclusively answer this question we will need to test the effectiveness of protamine-FokI fusion without the possibility of dimerisation in any other way than directed through random DNA binding by protamine.

Finally, non-repressible male sterility is not an ideal phenotype, from the perspective of mass rearing for use in the field. Because males are permanently

sterile, transgenic lines have to be maintained through constant backcrossing of transgenic females to wild type males, which prevents obtaining true breeding, homozygous line. Additionally, only half of the produced males carry the transgene. These shortcomings are absent in the conditional repressible system.

Chapter 5 – Conditional male sterility in *Ceratitis capitata*

5.1. Introduction

The experiments described in the previous chapter proved that expression of nucleases in the male germline is indeed capable of inducing severe sterility (paternal effect lethality) in transgenic males. However, it was still uncertain whether the protamine-FokI nuclease fusion protein can form active dimers by means of random binding of two fusion proteins adjacent to each other, without mediation of dimerisation through additional domains (like turboGFP).

This could be addressed via simple modifications of the constructs described in Chapter 3 – OX4466 and OX4467 – by removing tGFP from AeProtamine-tGFP-FokI fusion protein. Such a system, however, would still be non-repressible. In order to build a conditional, tetracycline repressible system we need to employ suitable promoters. As was mentioned before, the protamine promoter is very likely not appropriate in the context of a bipartite system, such as the tetracycline repressible expression system [8, 42]. This is because protamine is expressed late in spermatogenesis, with the mRNA transcript produced before the transcriptional shut-down during meiosis and translated later, after the completion of meiotic division. This, in turn, means that tTAV translated after transcriptional arrest may not be able to induce transcription of the protamine-nuclease effector.

At the time of designing experiments described in this chapter there were two potentially suitable promoters available in the Mediterranean fruit fly (Topi and β2-tubulin), but only one in *Aedes aegypti* (Topi). This was the reason for returning to *Ceratitis capitata* as a model organism in our next experiments. Furthermore, agricultural pests are the primary candidates for deployment of the sperm-sterile system, since early acting lethality prevents crop damage.

To further address the question of the feasibility of using a protamine-FokI (single cleavage domain) fusion as an effective DNA nuclease, an OX4458 effector construct was made. It contains a single FokI cleavage domain fused to a *Drosophila* protamine (with intron) under the transcriptional control of the tetO operator and it is a single ended piggyBac transposon with hr5-IE1 driven DsRed as a transformation marker. The OX4458 construct should not exert any effect on its own. Only when crossed to a suitable tTAV expressing line, in double heterozygotes, possessing both alleles, and in permissive conditions (without tTAV repressor – tetracycline) can expression of the effector fusion protein occur. A diagram showing structure of OX4458 construct is shown in *Figure 5.1*.

The protamine domain is supposed to target the whole fusion protein into the nucleus and ultimately to allow DNA binding. The intron within the protamine coding region – derived from the protamine gene itself – is to help in RNA-based expression tests. Omission of turboGFP removes the issue of the fluorescent tag facilitating dimerisation of the FokI cleavage domain independently from protamine DNA binding action – tGFP has to form a dimer to become active.

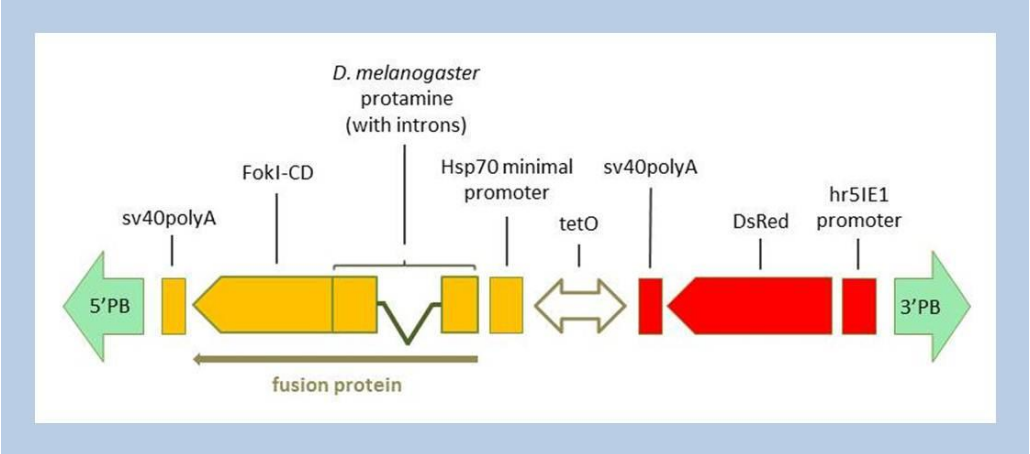


Figure 5.1 Schematic diagram of OX4458 construct. OX4458 construct contains fusion of *Drosophila melanogaster* protamine with FokICD under the control of tetO operator. When crossed with transgenic medfly lines expressing tTAV transactivator driven by sperm-specific promoter, resulting induction of protamine-FokICD expression should lead to sperm DNA cleavage and male sterility. The phenotype is conditional, repressible in the presence of tetracycline.

As was mentioned above, sperm specificity of tTAV (and ultimately the effector) expression was intended to be achieved by using medfly ß2-tubulin [57, 116, and 131] and Topi [93] promoters. If successful, Dm-Protamine-FokICD fusion would induce sterility / reduce egg hatch rates in permissive conditions upon induction by tTAV.

The β2-tubulin promoter has already been tried in *Ceratitis capitata* in OX4280 and OX4281 constructs, described in Chapter 3. These constructs were, however, based on the concept of GeneSwitch system, adding an additional layer of control over nuclease activity by a mechanism of reversible cytoplasmic sequestration of nuclease. As was argued in the Chapter 3, this additional complexity of the system caused difficulties in troubleshooting the whole system, if it did not perform as intended – as was the case.

Additionally, in the course of her DPhil project, Li Jin [61] showed that the same β2-tubulin promoter which was used in OX4280 and OX4281 was not capable of driving sperm-specific expression of either a fluorescent marker or I-PpoI homing nuclease in the bipartite, tetracycline repressible expression system. This promoter was, however, able to directly drive sperm-specific expression of fluorescent markers. It was hypothesised that this is due to the translation of the mRNA of β2-tubulin driven gene in late spermatogenesis stages, after the meiotic division. β2-tubulin driven tTAV is not able to function as a transcription factor in bipartite expression system after post-meiotic transcriptional shutdown.

Through a succession of promoter modifications Li Jin and colleagues were able to identify regulatory elements necessary and sufficient for successful use of *Ceratitis capitata* β2-tubulin promoter. *Figure 5.2* shows successive changes of the promoter and other regulatory elements leading to the final version of *Ceratitis capitata* β2-tubulin based promoter element – used in OX4282 tTAV expressing line.

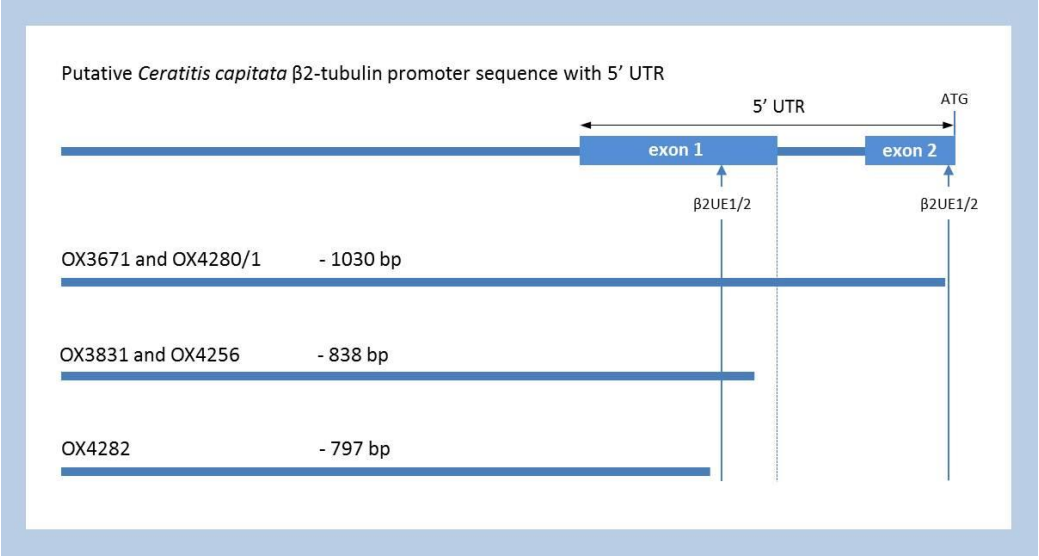


Figure 5.2 Different promoter fragments derived from *Ceratitis capitata* β2-tubulin promoter. β2UE1/2 motifs have been shown to be responsible for temporal- and tissue-specificity of the expression of the *Drosophila melanogaster* homologue**.**

Only after removing a large part of 5'-UTR, and replacing it with 5'-UTR from *Drosophila melanogaster* heat shock protein 83 (Dm-hsp83), was the modified β2-tubulin derived promoter in OX4282 construct able to drive strong, sperm-specific expression of fluorescent marker in the tetracycline-repressible bipartite system. *D. melanogaster* hsp83 expression starts earlier than that of β2-tubulin, in early primary spermatocytes, and continues throughout late spermatocytes stage until early elongation (Li Jin, DPhil Thesis [61]). The deleted fragment of β2-tubulin 5'-UTR contains, according to *in silico* analysis by Scolari *et al.* [110], two sets of β2UE1/2 motifs, which have been shown to be necessary for testis-specific expression of the *Drosophila melanogaster* homologue. It is possible that these elements are also responsible for translational delay of β2-tubulin mRNA until after meiosis. Replacing them with 5'-UTR from *Drosophila melanogaster* hsp83 might have shifted translation of tTAV in OX4282 lines earlier, enabling in turn pre-meiotic transcriptional activation of elements under the control of tetO operator.

Apart from these modifications of promoter driving tTAV expression, OX4282, the piggyBac transposon based construct contains also turboGFP under the control of tetO operator, as a reporter of tTAV expression, and hr5iE1-driven DsRed as the transformation marker, as shown below in *Figure 5.3*.

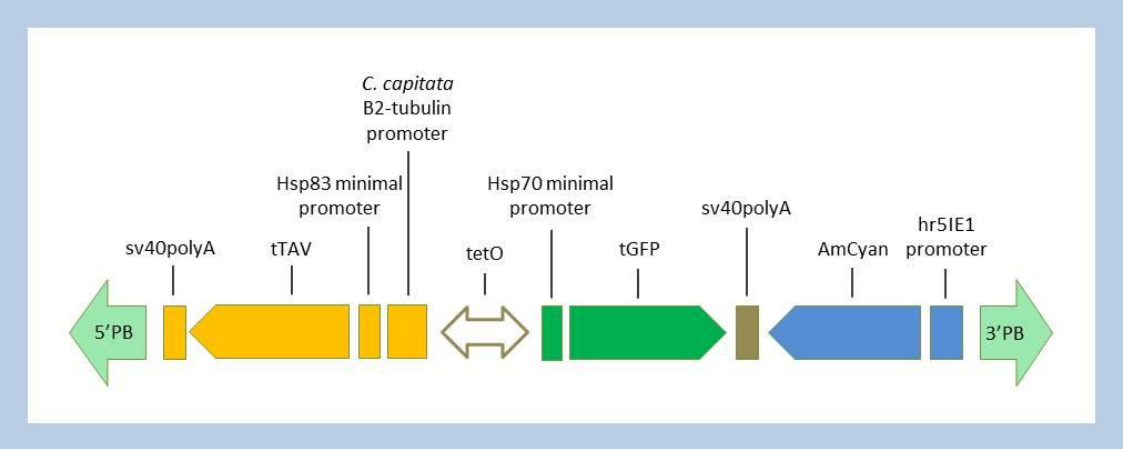


Figure 5.3 Schematic diagram of OX4282 construct. OX4282 transposon contains tTAV coding sequence under the control of the fragment of Mediterranean fruit fly β2-tubulin promoter. The fragment used contains most upstream 797 bases of the putative β2-tubulin promoter. Both β2UE1/2 motifs were replaced with hsp83 minimal promoter derived from *Drosophila melanogaster*. TurboGFP coding sequence, under the control of tetO operator, was included as a reporter of tTAV expression.

After Li Jin and Romisa Asadi demonstrated sperm-specific expression of effectors in crosses with the OX4282 promoter lines (personal communication), one of these lines was chosen to test induction of male-specific sterility caused by germline expression of protamine-FokI fusion.

A second male germline promoter considered potentially suitable for our conditional sperm-sterility system was matotopetli (Topi). The initial attempts to isolate a functional Topi promoter fragment were unsuccessful. An 1178 bp fragment of putative Topi promoter from *Ceratitis capitata* failed to drive sperm-specific expression of markers (or other effectors), both in direct expression and in a bipartite "tetO system" configuration. Later, a larger, 1709 bp fragment was selected, with additional 539 bp which includes 484 bp of possible Topi coding sequence and a 55 bp intron. This promoter fragment was used in OX4391 construct to drive germline expression of the tTAV transactivator. The inclusion of part of the Topi coding sequence would cause addition of extra amino acids fragment to tTAV protein, which might disrupt the function of the tTAV protein. To

avoid this, an ubiquitin sequence was inserted in frame between the coding sequences of Topi and tTAV. This should result in post-translational separation of two protein domains / fragments through cleavage by ubiquitin protease, leaving the tTAV transactivator without any fused peptide fragments [124, 125].

Like OX4282, OX4391 contains a tTAV expression reporter in the form of turboGFP under the control of a tetO operator plus AmCyan as the transformation marker. Details of OX4391 transposon design are shown in *Figure 5.4*.

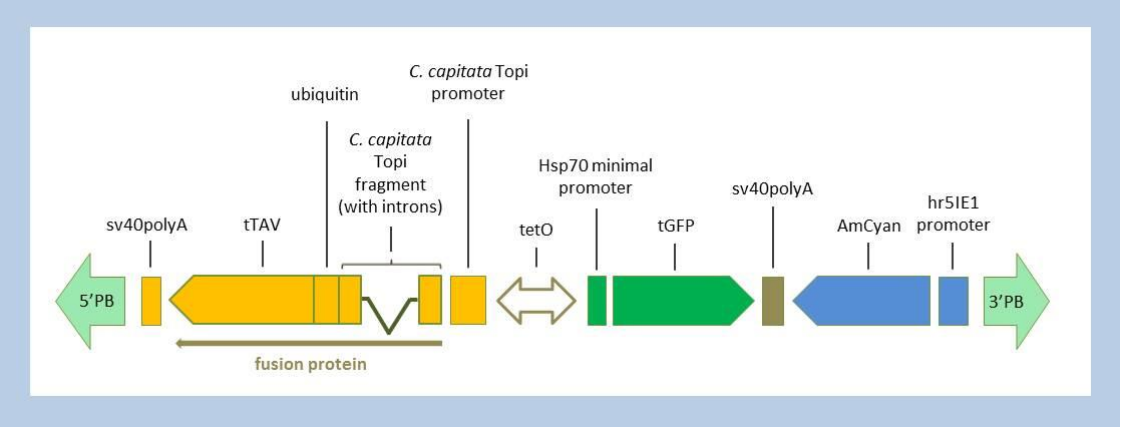


Figure 5.4 Schematic diagram of OX4391 construct. OX4391 transposon contains tTAV transactivator under the control of 1709 bp fragment derived from *Ceratitis capitata* matotopetli (Topi) 5' regulatory region. This fragment includes putative Topi promoter and a fragment of Topi coding sequence with intron. Ubiquitin located immediately upstream of the tTAV is used to post-translationally cleave expressed tTAV-Topi fragment fusion leaving tTAV without unwanted amino acid tags. TurboGFP coding sequence, under the control of tetO operator, was included as a reporter of tTAV expression.

Crosses between transgenic lines carrying OX4391 transposon and transgenic lines expressing fluorescent marker under the control of tetO operator resulted in tetracycline-repressible sperm-specific expression of marker [61].

Development of Topi promoter from *Ceratitis capitata*, culminating in the design of OX4391 transposon and generation of transgenic Mediterranean fruit fly lines, was performed by Li Jin and other colleagues at Oxitec. One line, OX4391H, was used in tests of induction of male germline expression of protamine-FokI nuclease fusion described in this chapter.

5.2. Results

5.2.1. Obtaining OX4458 lines (tetO-DmProtamine-FokICD)

OX4458 plasmid was co-injected into pre-blastoderm medfly embryos with OX3022 helper plasmid as the source of piggyBac transposase. 587 eggs were injected in total. Post-injection survival was as follows:

187 larvae → 152 pupae → 139 adults (79 males + 60 females)

Further crosses were performed as described before. G_2 and G_3 adults were tested for wild type to transgenic and male to female ratios as an indication of single versus multiple insertions and autosomal versus sex chromosome insertion respectively.

Four lines (B1, D1, D2, and F2) were chosen to be kept out of all 6 and were used in subsequent experiments.

OX4458-B1	212 wild type	(109 ♀ + 103 ♂)	and	190 transgenic	(85 ♀ + 105 ♂)
OX4458-C1	182 wild type	(♂ only)	and	155 transgenic	(♀ only)
OX4458-D1	181 wild type	(86 ♀ + 95 ♂)	and	189 transgenic	(96 ♀ + 93 ♂)
OX4458-D2	162 wild type	(86 ♀ + 76 ♂)	and	149 transgenic	(65 ♀ + 84 ♂)
OX4458-F2	147 wild type	(76 ♀ + 71 ♂)	and	186 transgenic	(97 ♀ + 89 ♂)
OX4458-J1	22 wild type	(11 ♀ + 11 ♂)	and	22 transgenic	(8 ♀ + 14 ♂)

Table 5.1 Phenotype segregation in OX4458 *Ceratitis capitata* lines. Wild type to transgenic and male to female ratios in G_3 of OX4458 lines were assessed to select lines with single, autosomal insertion exhibiting strong expression of fluorescent marker. Four lines, B1, D1, D2 and F2, were ultimately chosen for continued breeding.

5.2.2. The design of hatch-rate assays

The design of the experiment is shown on *Figure 5.5*. Effector (E) and Promoter (P) lines were crossed. Eggs from these crosses are collected and divided into either permissive conditions (without tetracycline) or repressive conditions (with tetracycline) group and reared accordingly. Pupae are screened for expression of line-specific fluorescent markers and double heterozygotes – with both markers – collected. These were checked for equal male to female ratio after eclosion and males crossed to wild type females – again either on- or off-tet. Double-heterozygote females to wild type males crosses were used to test whether any observed effects are sex specific. A wild type control was included as the reference point for egg hatch rates. Eggs from the crosses were collected and counted. Three days later counting was repeated and the numbers of not hatched eggs recorded. The hatching rate is then calculated as the percentage of hatched eggs in each sample (progeny of the cross), with collected eggs representing 100%.

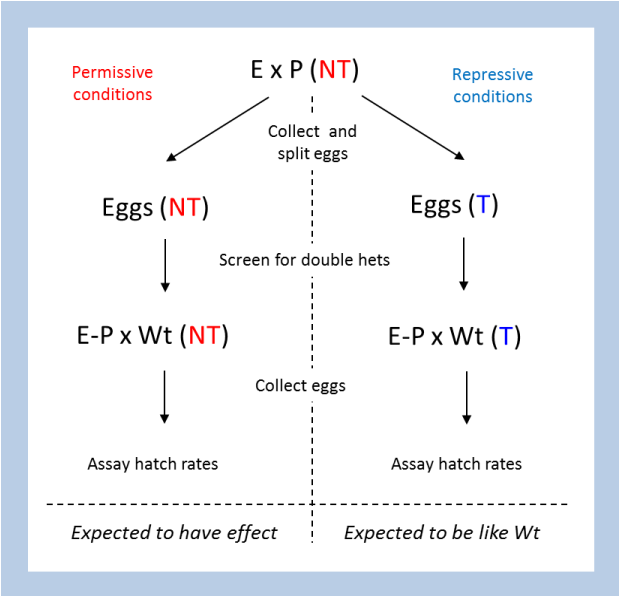


Figure 5.5 Schematic diagram of hatch-rate assay design. Permissive conditions (NT) – individuals were reared on a diet without tetracycline; Repressive conditions (T) – individuals were reared on a diet with tetracycline.

5.2.3. β2-tub-tTAV-driven expression of Dm-Protamine-FokICD

5.2.3.1. OX4282L and OX4458 crosses

To test the suitability of a β2-tublin promoter fragment from *Ceratitis capitata* for use in our conditional male-sterility system all four established OX4458 lines (B1, D1, D2, and F2) were crossed to OX4282-L (β2-tubulin-tTAV) promoter line. Progeny of these crosses, reared either in the presence or absence of dietary tetracycline, was screened for the expression of DsRed – OX4458 lines – and AmCyan – OX4282L, and double-heterozygotes collected. Observed segregation of different fluorescent phenotypes confirmed a single insertion of each transgene and similar ratios of male to female in enclosed adult double heterozygotes their autosomal localisation, as shown in *Table 5.2*.

G1; NT	**Red**	**Cyan**	**WT**	**Red+Cyan**	**females**	**males**
4458-B1x4282-L	94	103	103	95	35	51
4458-D1x4282-L	151	126	152	105	38	48
4458-D2x4282-L	140	130	137	155	70	63
4458-F2x4282-L	183	140	186	114	34	35
G1; T						
4458-B1x4282-L	66	66	75	55	7	15
4458-D1x4282-L	74	66	72	60	21	26
4458-D2x4282-L	55	49	75	45	19	22
4458-F2x4282-L	76	66	58	66	25	31

Table 5.2 Screening of the fluorescence in the progeny from crosses of OX4458 effector lines to OX4282-L promoter line. Upper panel – off-tetracycline; lower panel – on-tetracycline. On the right – number of males and females eclosed from collected double-heterozygote pupae. Phenotype segregation confirms single, autosomal insertions of transgenic alleles.

In some double heterozygotes there is some deviation from equal ratios of each fluorescent phenotype. This is most likely due to errors in screening – underscoring the number of pupae with AmCyan marker. Fluorescence of the AmCyan marker is much weaker than DsRed fluorescence; this makes it more difficult to positively identify pupae that are expressing AmCyan, especially when pupae are older, when the puparium thickens and darkens. Additionally, DsRed fluorescence may mask AmCyan; although in that case the effect would be most pronounced in the case of lines D2 and F2 as they demonstrate the strongest DsRed fluorescence as shown in *Figure 5.6*.

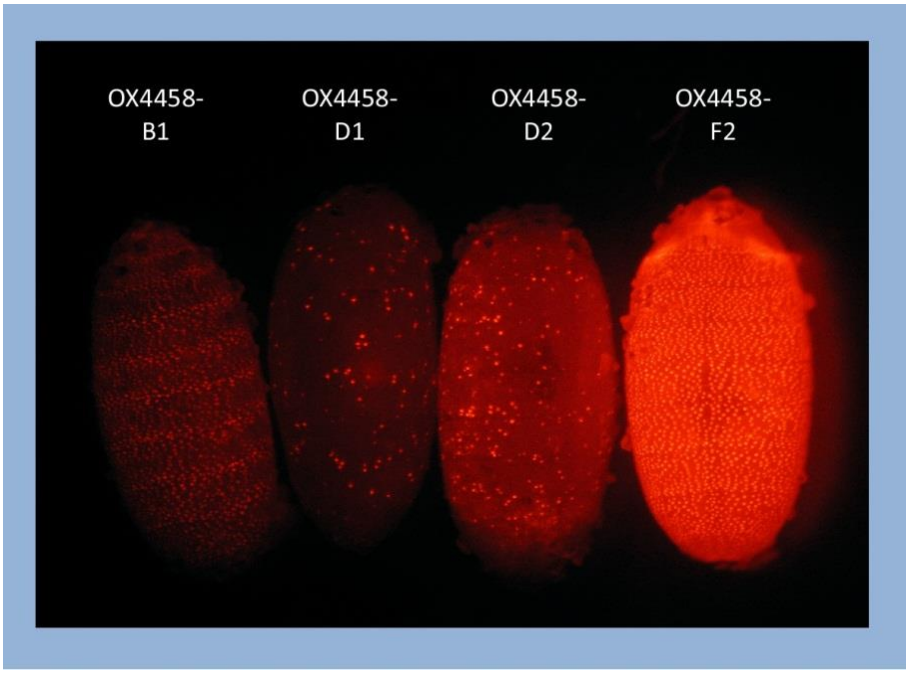


Figure 5.6 Relative levels of DsRed (transformation marker) expression in different OX4458 lines. Example of positional effect – dependence of transgene pattern of expression and expression levels on transcriptional state of chromatin adjacent to transgene insertion site.

For the hatch-rate assay double heterozygote males carrying both Ccβ2-tubulin-tTAV and tetO-DmProtamine-FokI alleles were crossed with wild type females. Wild type males crossed to wild type females were used as the control. An

additional control of OX4282-L males crossed to wild type females was included – to rule out possible influences on male sterility caused only by testis-specific expression of tTAV transactivator.

All crosses were done both in permissive conditions (without tetracycline) and in repressive conditions (with tetracycline). Four egg collections were carried out from each cross. Percentages of unhatched eggs were calculated for each collection, and then mean percentage of hatch-rate was obtained. Therefore, degree (percentage) of sterility of each line (genotype) in particular conditions was obtained.

Results show a striking effect of activation of nuclease with a ß2-tubulin driven tTAV – almost 100% sterility of double-heterozygote males in permissive conditions [*Fig 5.7*]. Sterility is observed with all four different OX4458 insertions suggesting little position effect in this particular situation. Sterility of double-heterozygote males in the presence of a repressor remains at the same level as in wild type males reared on tetracycline – meaning that the effect is tetracycline dependent. There is also no increased sterility on tetracycline in OX4282-L males crossed to wild type females. All of the above strongly suggests that the sterility seen in this assay is due to action of both components of the system, meaning that it is the result of the activation of nuclease expression by tTAV and subsequent sperm DNA cleavage.

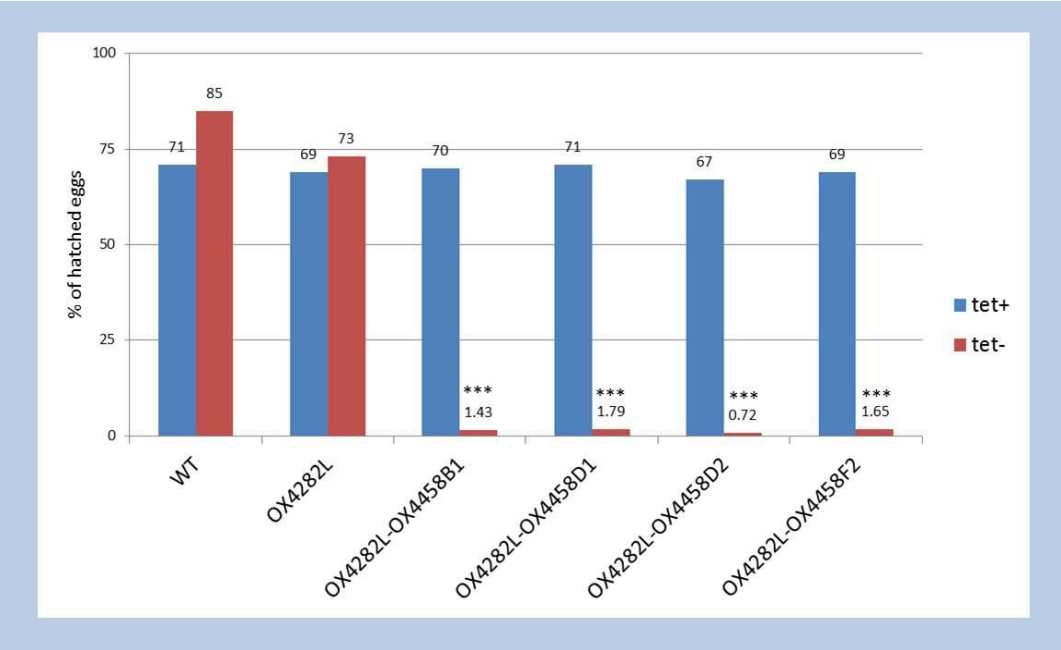


Figure 5.7 Repressible male sterility in *Ceratitis capitata* lines carrying both ß2-tubulin-tTAV (OX4282L) and tetO-DmProtamine-FokI (OX4458) alleles. Males carrying both driver and effector alleles, reared and bred either on a diet with (tet+) or without tetracycline (tet-), were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type and OX4282L male crosses with wild type females in the presence or absence of tetracycline were used as controls. Crosses where highly significant male sterility was observed (chi-squared test, P<0.0001) are marked with asterisks.

To test reproducibility of the observed male sterility phenotype, the hatch-rate assay was repeated. Only two OX4458 lines were used this time – D2 and F2. Additionally, transgenic females carrying both OX4282L and OX4458D2 (or F2) alleles were crossed to wild type males as a control for sex-specificity of the phenotype. Results, shown in *Figure 4.8*, confirmed previously obtained data, and proved male-specificity of the nuclease expression, as hatch-rates in progeny from both female crosses did not seem effected.

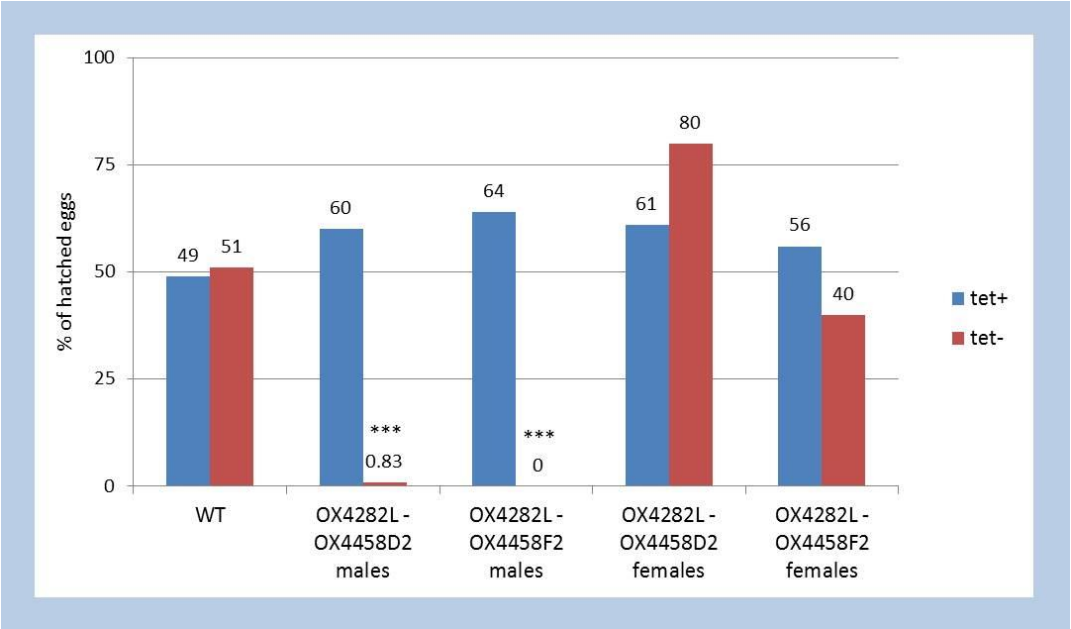


Figure 5.8 Male-specificity of the sterility phenotype in *Ceratitis capitata* lines carrying both ß2-tubulin-tTAV (OX4282L) and tetO-DmProtamine-FokI (OX4458) alleles. Males or females carrying both driver and effector alleles, reared and bred either on a diet with (tet+) or without tetracycline (tet-), were crossed to the wild type and the hatching rates of eggs obtained from these crosses were calculated. Wild type and OX4282L male controls were included. Crosses where highly significant male sterility was observed (chi-squared test, P<0.0001) are marked with asterisks.

5.2.3.2. OX4483 and OX4458 crosses

The OX4282L β2-tubulin promoter-tTAV line used in above experiments contains, besides transformation marker, an additional fluorescent protein expression cassette – tetO controlled turboGFP. This serves as an internal control for tTAV expression – in permissive conditions tTAV binds to its operator and activates the expression of tGFP thus signalling its proper functioning. The proximity of the tetO operator to the β2-tubulin promoter and tTAV transactivator may, however, result in self-amplification of tTAV mRNA message and increase of intracellular levels of tTAV protein in a positive feed-back loop manner. Such build-up of tTAV can in itself cause high cells toxicity [40]. On the other hand, higher levels of tTAV

expression can drive stronger expression of the effector, influencing phenotype penetrance.

The OX4282L male control did not show much evidence for such effect, but this could be the result of position effect, or an assay artefact. To address the issue of potential tTAV positive feed-back loop formation, the tetO-turboGFP reporter element was removed from OX4282 constructs resulting in OX4483 transposon, shown in *Figure 5.9*.

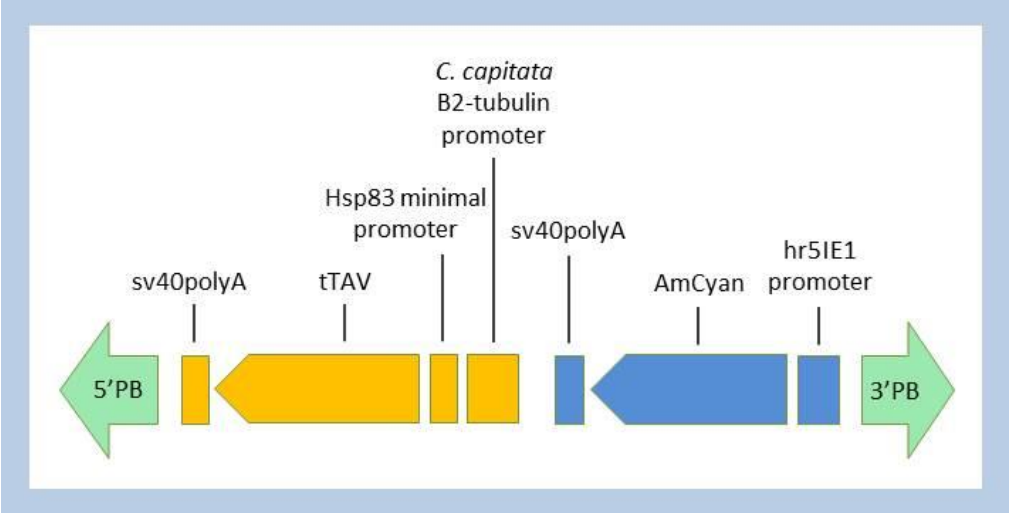


Figure 5.9 Schematic diagram of OX4483 construct. OX4483 transposon is similar to OX4282 transposon in that it also contains tTAV coding sequence under the control of the fragment of the Mediterranean fruit fly β2-tubulin promoter. The fragment used contains most upstream 797 bases of the putative β2-tubulin promoter. Both β2UE1/2 motifs were replaced with hsp83 minimal promoter derived from *Drosophila melanogaster*. However, turboGFP coding sequence, under the control of tetO operator, was removed to minimise the risk of formation of positive feed-back loop.

Four independent OX4483 transgenic lanes were obtained (Romisa Asadi and Michal Bilski) – A, Da, E, and Q. These were crossed to OX4458-B line, to obtain individuals carrying both alleles [*Table 5.3*].

G1; NT	Red	Cyan	WT	Red+Cyan
OX4483A x OX4458B	86	93	92	93
OX4483Da x OX4458B	24	24	27	23
OX4483E x OX4458B	42	32	40	32
OX4483Q x OX4458B	25	13	23	22
G1; T				
OX4483A x OX4458B	106	102	98	106
OX4483Da x OX4458B	30	31	40	34
OX4483E x OX4458B	36	24	30	31
OX4483Q x OX4458B	33	33	28	38

Table 5.3 Fluorescent phenotypes in the progeny from crosses of OX4458-B effector line to OX4483 promoter lines. Upper panel shows crosses performed in the absence of tetracycline; lower panel shows crosses performed in the presence of tetracycline. . Phenotype segregation confirms single, autosomal insertions of transgenic alleles.

Double heterozygous males carrying both OX4458 and OX4483 transposons were crossed to wild type females and resulting eggs assayed for hatch-rate. Wild type and OX4458 controls were included. As shown in *Figure 5.10*, embryos fathered by double heterozygous males which were reared without tetracycline, have reduced hatch-rate compared to ones fathered by on-tet reared controls. The effect, however, was varied between the lines, ranging from practically negligible in OX4483A – OX4458B males to almost complete sterility in OX4483Q – OX4458B males. This indicates that very strong sterile phenotype penetrance can be achieved without the tTAV positive feed-back loop.

However, since only one OX4282 line was analysed in crosses with tetO-DmProtamine-FokI effector it is not certain whether such strong position effect is reduced in lines in which self-amplification of tTAV can occur.

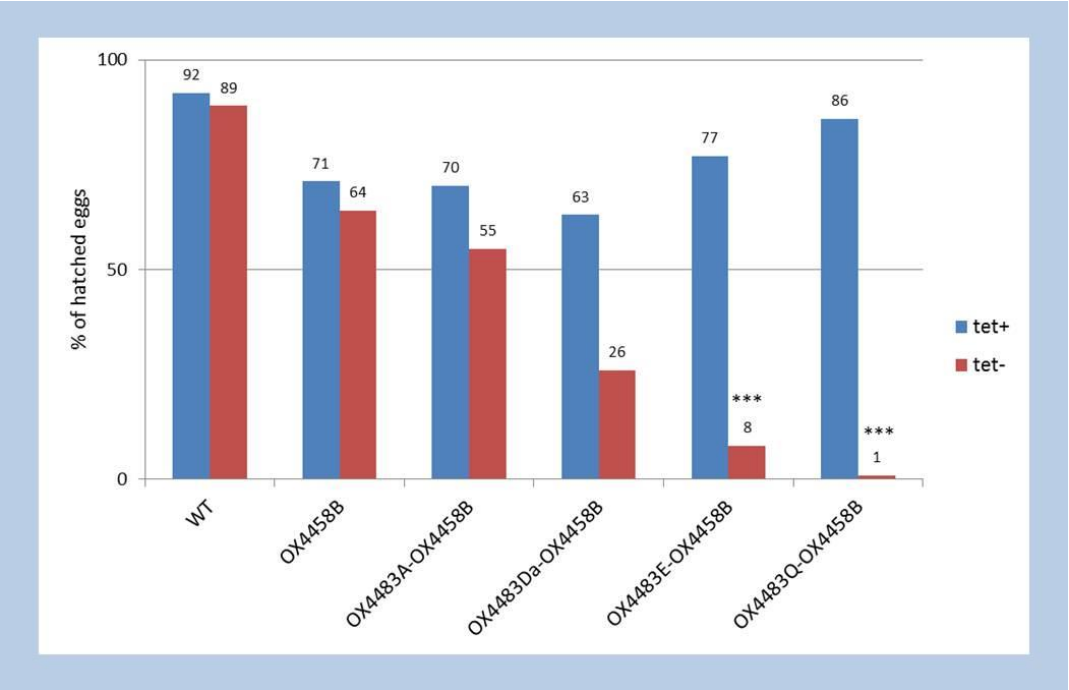


Figure 5.10 Repressible male sterility in *Ceratitis capitata* lines carrying both OX4483 and OX4458 alleles. Males carrying both driver and effector alleles, reared and bred either on a diet with (tet+) or without tetracycline (tet-), were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated. Wild type and OX4458 controls were included. Crosses where highly significant male sterility was observed (chi-squared test, P<0.0001) are marked with asterisks.

5.2.4. Topi-tTAV-driven expression of Dm-Protamine-FokICD (OX4391H and OX4458 crosses)

To test whether the second promoter of choice – Topi – can successfully drive sperm-specific expression of nuclease in a tTAV/tetO conditional system, a similar hatch-rate assay was performed using OX4391-H line expressing Topi driven tTAV. OX4391-H line and all four OX4458 lines were crossed to produce males carrying both driver and effector alleles [*Table 5.4*].

G1; NT	Red	Cyan	WT	Red+Cyan	females	males
4458-B1x4391-H	209	211	211	186	56	49
4458-D1x4391-H	124	121	155	100	38	36
4458-D2x4391-H	124	147	116	115	27	27
4458-F2x4391-H	64	67	88	77	27	28
G1; T	**Red**	**Cyan**	**WT**	**Red+Cyan**	**females**	**males**
4458-B1x4391-H	44	66	68	45	19	17
4458-D1x4391-H	61	51	55	62	21	19
4458-D2x4391-H	30	36	41	35	11	17
4458-F2x4391-H	66	58	43	58	14	22

Table 5.4 Fluorescent phenotypes in the progeny from crosses of OX4458 effector lines to OX4391H promoter line. Upper panel shows crosses performed in the absence of tetracycline; lower panel shows crosses performed in the presence of tetracycline. Phenotype segregation confirms single, autosomal insertions of transgenic alleles.

Double heterozygous males, tetracycline fed or ”tet-free”, were crossed to wild type females. Controls consisting of wild type males or OX4391H males crossed to wild type females were also performed.

Collected data shows that activation of a nuclease with Topi driven tTAV was less successful compared to performance of the β2-tubulin promoter [*Fig 5.11*]. There was very little difference in hatch rates between eggs fathered by double-heterozygote males or wild type males, both on- or off-tet. The experiment was repeated with two OX4458 lines – D2 and F2 – this time including also transgenic females carrying both alleles as sex-specificity control. The difference in percentages of hatched eggs between off-tet samples and on-tet or wild type control was again negligible [*Fig 5.12*].

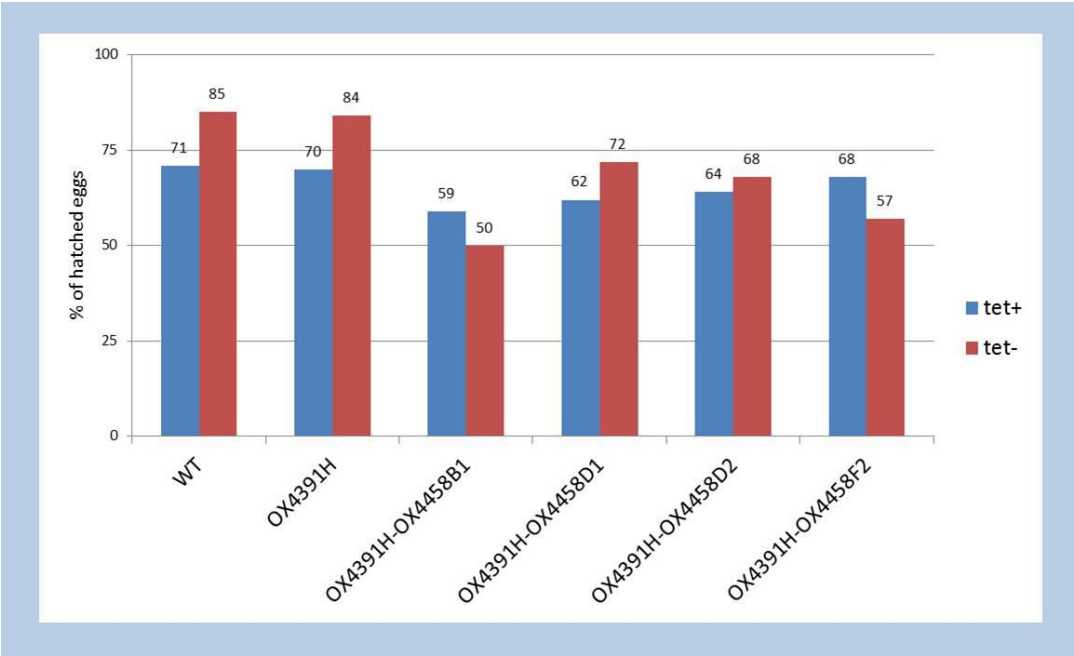


Figure 5.11 1st male sterility assay in *Ceratitis capitata* lines carrying both OX4391 and OX4458 alleles. Males carrying both driver and effector alleles, reared and bred either on a diet with (tet+) or without tetracycline (tet-), were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated. Wild type and OX4391H controls were included.

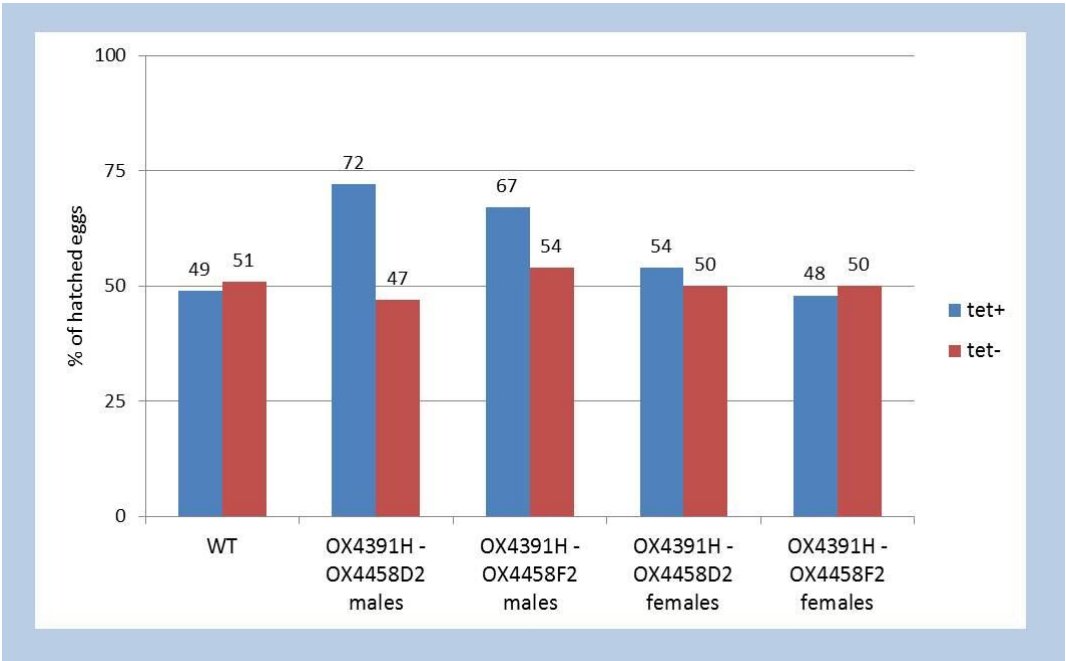


Figure 5.12 2nd sterility assay in *Ceratitis capitata* lines carrying both OX4391 and OX4458 alleles. Males or females carrying both driver and effector alleles, reared and bred either on a diet with (tet+) or without tetracycline (tet-), were crossed to the wild type and the hatching rates of eggs obtained from these crosses were calculated. Wild type controls was included.

5.3. Conclusions

The results of the OX4458 crosses with tTAV expressing lines proved finally that sperm-specific expression of protamine-FokI fusion protein can induce male sterility in conditional, bipartite expression system. No mediation of dimerisation of protamine-FokI through additional domains is required. This suggests that non-specific DNA binding by protamine in the protamine-FokICD fusion can randomly happen close enough for two molecules to help them form a dimer and thus activate FokI nuclease. Our results proved that one of two available medfly promoters – ß2-tubulin – works and can be employed in the system.

It is not clear why the expression of tTAV driven by the second promoter (Topi) failed to induce sterility in combination with tetO-nuclease lines. One probable explanation is due to the Topi promoter being considered weaker than the β2-tubulin promoter (*personal communication by Dr Helen White-Cooper*) it may not able to express Protamine-FokI fusion in quantities sufficient enough to promote dimer formation. The nuclease has to form a dimer to be active and this is facilitated only by nonsequence-specific DNA binding by protamine. The probability of two such fusion proteins coming into contact with each other is directly dependent on the concentration of the fusion protein – derivative of expression levels / promoter strength – and below some threshold level virtually negligible. Such a mechanism provides additional protection against leakiness of promoters used in the system. If this explanation is true then it would mean that the system is indeed working as envisioned. Other experiments in Oxitec, in which Topi-tTAV driven activation of sequence specific IPpoI homing endonuclease induced noticeable male sterility (data not included), corroborate this explanation.

Additionally, it is possible that the selected fragment of endogenous *Ceratitis capitata* is not capable of driving maximal expression of tTAV at the right time and place. Selecting a different fragment and introduction of modifications similar to ones carried out in case of β2-tubulin promoter could improve promoter performance.

Lastly, the inability of Topi promoter to induce sterility in conjunction with tetO-Protamine-FokI nuclease could be the result of position effect. The OX4391H line chosen for experiments based on its performance in crosses with fluorescent marker reporter lines and effector lines containing I-PpoI nuclease under the control of tetO operator (experiments performed by Li Jin and Romisa Asadi) as a best line from the panel of several available ones. Nevertheless, position effect cannot be completely ruled out until results obtained with several other lines disprove such explanation.

Chapter 6 – Conditional male sterility in *Aedes aegypti*.

6.1. Introduction

Previous experiments proved the feasibility of using expression of a DNA nuclease in the male germline to cause severe male sterility (paternal-effect lethality) both in *Aedes aegypti* and in *Ceratitis capitata*. The system described in Chapter 4 for *Aedes aegypti* was constitutive and non-repressible. The major drawback of such a system is the lack of ability to turn the expression of the nuclease off – it is always "on" and hence transgenic males are always sterile. This necessitates the maintenance of transgenic lines through females and, more importantly, excludes the possibility of obtaining homozygous, true breeding lines. The next step, therefore, was to create a conditional male sterile system in *Aedes aegypti*.

For building such a conditional system in mosquitoes the researcher decided to draw on the results obtained in Mediterranean fruit fly and test matotopetli (topi) and β2-tubulin promoters as drivers for the expression of tTAV transactivator.

Aedes aegypti topi was already tested in Oxitec's laboratories as a male-germline-specific promoter, and driver (topi-tTAV) lines were available in the form of OX4286-Ae lines – piggyBac transposon based construct OX4286 contains tTAV driven by *Aedes aegypti* derived topi promoter and topi 5'UTR. The tTAV coding sequence is followed by a 3'UTR element from *Drosophila melanogaster* fs(1)K10. This is based on the successful prior use of this sequence in *Drosophila*

melanogaster, where it was used in pUASp as one of several changes to the standard UAS vector pUAST to enhance expression in the female germline [100]. OX4286 employs 3xP3 driven DsRed as a transformation marker. 3xP3 is an artificial, eye-specific promoter, responsive to the evolutionary conserved Pax-6 transcription factor (*Drosophila melanogaster eyeless*) and is active during embryonic, larval and pupal stages [12, 54]. The previously tested line OX4286B was used in experiments described further.

To test the activity of the Topi promoter, a reporter line, OX3979-Ae, was used. The OX3979-Ae contains an AmCyan coding sequence under the control of tetO operator, integrated into a genomic docking site using the phage C31 system [87]. It expresses hr5IE1 driven DsRed as the transformation marker. Double heterozygotes carrying both Topi-tTAV and tetO-AmCyan, generated by crossing together OX4628B and OX3979, showed clear expression of AmCyan in *Aedes aegypti* testis from later larval stages, as shown in *Figure 6.1*.

To test the suitability of the β2 tubulin promoter for use in the conditional male sterility system in *Aedes aegypti* mosquitoes, OX4635, a piggyBac -based construct was built.

Smith *et al*., in their 2007 paper, described cloning and characterisation of the *Aedes aegypti* β2-tubulin promoter and defined its 959 bp fragment as sufficient for driving DsRed expression in mosquito testis – in stage and tissue-specific manner similar to endogenous promoter. This represented successful direct expression of a reporter gene and was similar in terms of design to our previously tested constitutive male sterile system. To adapt the promoter for use in our conditional expression system we decided to remove as far as possible the transcribed sequences of β2-tubulin, reasoning that these were likely to mediate

the translational delay typical of β2-tubulin but which would highly undesirable for the proposed bipartite expression system (this issue is discussed in the context of Medfly in Chapter 5) The 5'UTR, along with first 36 bp of ORF present in the sequence used by Smith *et al.* (2007), was removed and replaced with an hsp83 minimal promoter from Mediterranean fruit fly [*Fig. 6.2*]. This altered promoter was employed to drive tTAV expression in construct OX4635. OX4635 contains also hr5IE1 – driven AmCyan as the transformation marker [*Fig. 6.2*].

To test the second part of the conditional sterility system two constructs, with Protamine-FokI Cleavage Domain fusions under the control of tetO operator, were used. One of these, OX4458, had previously been tested in *Ceratitis capitata* with very promising results, though it was not known whether the *Drosophila melanogaster* protamine used in this construct to target FokI nuclease cleavage domain (FokICD) onto DNA would work in *Aedes aegypti*.

The second construct, OX4627, is very similar in overall design to OX4458 – both are piggyBac-based, both use hr5EI1-driven DsRed as the transformation marker, but there are also important differences.

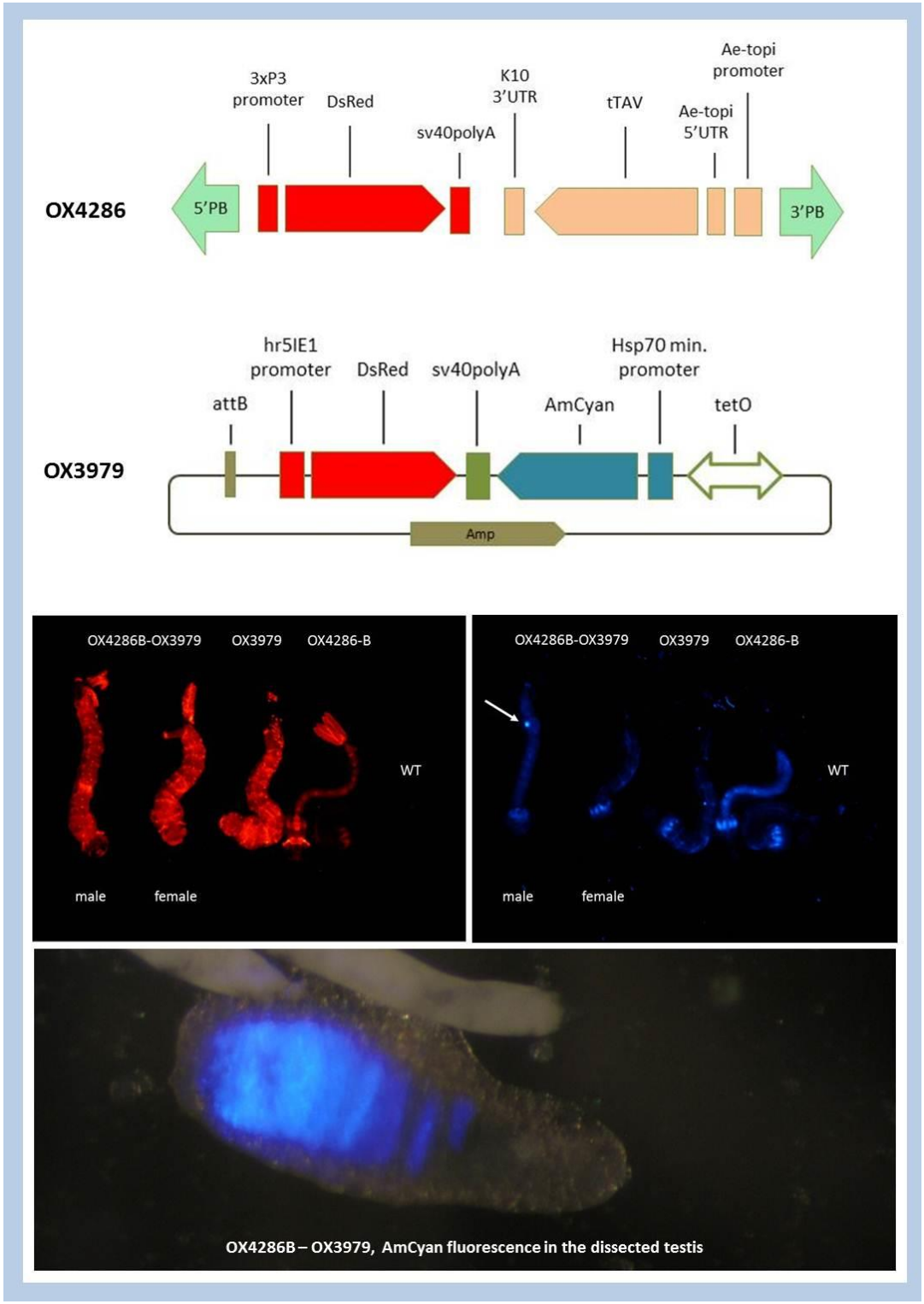


Figure 6.1 Topi-tTAV driven expression of AmCyan in *Aedes aegypti* testis. Schematic diagrams of OX4286 and OX3979 constructs. Upper images show *Aedes aegypti* larvae of different genotypes seen under red fluorescence filter (left), or blue fluorescence filter (right). AmCyan expression in the testis of male OX4286B-OX3979 double heterozygote is marked with the white arrow. Lower image (courtesy of Derric Nimmo, Oxitec Ltd.) shows AmCyan fluorescence in the dissected testis of double heterozygote OX4286B – OX3879 male.

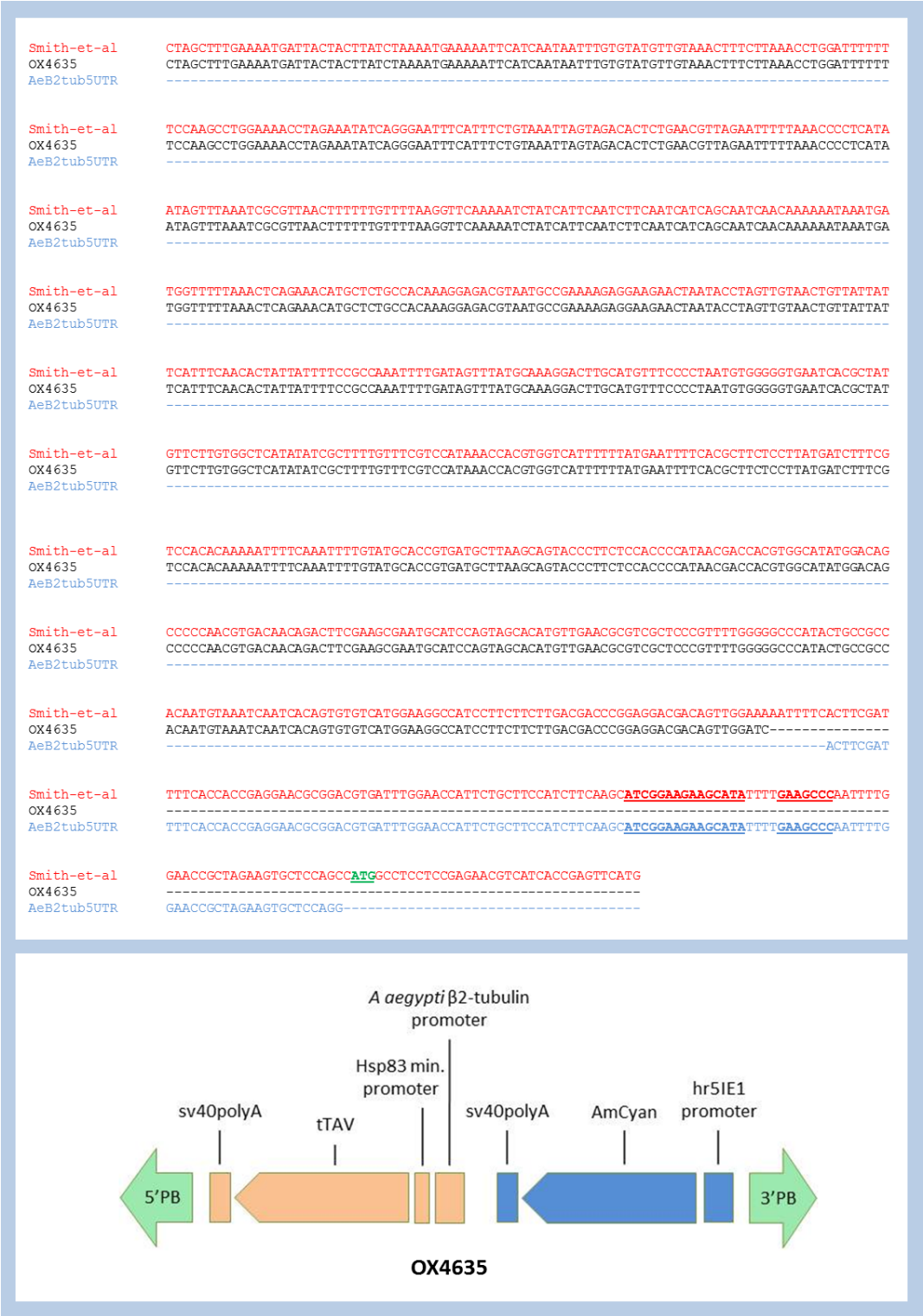


Figure 6.2 *Aedes aegypti* β2-tubulin promoter modifications in the OX4635 construct. Upper panel: alignment of sequences of β2-tubulin promoters from *A. aegypti*: in red – sequence of the promoter by Smith *et al*. [116]; in black – sequence of promoter as used in OX4635 transposon; in blue – sequence of *A. aegypti* β2-tubulin 5'UTR from DQ833526 entry in GeneBank. In underlined bold red and blue – B2UE1 and β2UE2 motifs; β2-tubulin ORF start marked in bold, underlined green. Lower panel: schematic diagram of OX4635 construct.

To target the FokI nuclease cleavage domain onto the DNA, OX4627 uses *Aedes aegypti* protamine (AeProtamine), as was successfully used in OX4466 and OX4467 constructs constitutively expressing nucleases in mosquito testes. Since the *Aedes aegypti* protamine sequence used in these constructs does not contain any introns, we used an intron from the *Drosophila melanogaster* alcohol dehydrogenase gene (DmAdh). A similar intron element has been previously tested, and shown to work, in Oxitec's laboratories as part of a different system (A. Collado personal communication).

The reason for the addition of intron in OX4627 was twofold. Firstly, it was used to facilitate possible future RT-PCR analysis of transgene expression. Secondly, the construct was planned as a sort of template or chassis for designing constructs for testing potential intron candidates for sex-specific splicing – as an alternative way of achieving sex-specific expression of effectors.

Since the alcohol dehydrogenase exon fragments surrounding the intron add extra amino acids to the translated AeProtamine – FokICD fusion protein, an ubiquitin moiety was been added between DmAdh exon 4 and AeProtamine. When translated, the ubiquitin directs sequence-specific cleavage of the fusion protein immediately C-terminal to the ubiquitin leaving AeProtamine-FokICD fusion without any extra amino acids [124, 125].

Schematic diagrams of OX4458 and OX4627 transposons with details of fusion protein design in the latter are shown in *Figure 6.3*.

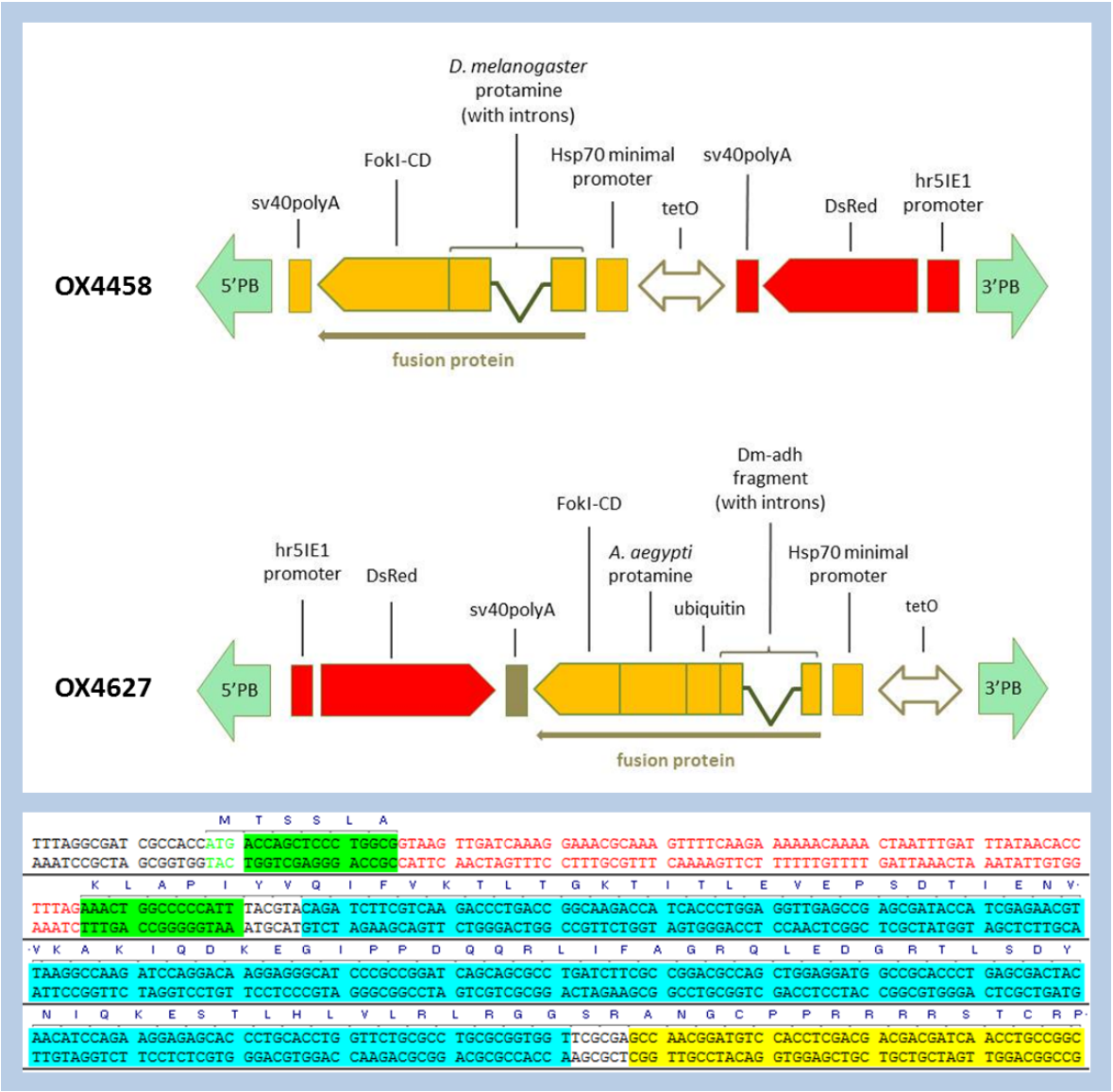


Figure 6.3 Comparison of tetO-Protamine-FokI effector constructs tested in *Aedes aegypti*. Upper panel shows schematic diagrams of OX4458 and OX4627 constructs. Both transposons contain the fusion of *Drosophila melanogaster* (OX4458) or *Aedes aegypti* (OX4627) protamine with FokICD under the control of tetO operator. OX4458 and OX4627 transposons were supposed to lead to *Aedes aegypti* conditional (repressible) male sterility in crosses with transgenic lines expressing tTAV transactivator driven by sperm-specific promoter. Lower panel shows details of protein fusion of AeProtamine with FokI Cleavage Domain in OX4627 transposon: in red font – *Drosophila melanogaster* alcohol dehydrogenase gene (DmAdh) intron 4; green blocks – fragments of DmAdh exons 3 and 4 respectively; blue block – ubiquitin; yellow block – fragment of *Aedes aegypti* protamine.

6.2. Results

6.2.1. Obtaining transgenic lines

6.2.1.1. OX4458

OX4458 plasmid was co-injected into pre-blastoderm *Aedes aegypti* embryos together with piggyBac transposase mRNA prepared from OX3081 plasmid – as described in Materials and Methods. Two sets of injections were performed with 1456 eggs and 635 eggs injected correspondingly. Post-injection survival was as follows:

1456 eggs → 64 larvae → 11♀ + 16 ♂ (pupae) → 9 ♀ + 16 ♂ (adults)
635 eggs → 204 larvae → 81♀ + 89 ♂ (pupae) → 74 ♀ + 80 ♂ (adults)

Surviving adults were crossed to wild type in 12 G0 crosses. Transformants were found among progeny of 10 of these crosses. These were crossed to wild type in single male crosses to obtain separate insertion lines. G2 progeny were analysed for transgenic versus non-transgenic (wild type) and male versus female ratios, as shown in *Table 6.1.* Lines marked in bold were propagated for the next generations.

From G4 generation only lines marked in bold were maintained and used in further experiments [*Table 6.2*]. Samples from OX4458-F1 lines were frozen for future analysis of insertion site as it seems that this is a potential male-specific insertion.

G1 cross	G2 pupae transgenic		wild type		G1 cross	G2 pupae transgenic		wild type	
	♀	♂	♀	♂		♀	♂	♀	♂
OX4458-A1	**51**	**47**	**59**	**43**	**OX4458-G1**	**43**	**33**	**42**	**42**
OX4458-A2	23	62	57	61	OX4458-G2	11	2	7	3
OX4458-B1	13	39	24	34	OX4458-H1	24	29	37	30
OX4458-B2	**16**	**18**	**14**	**11**	**OX4458-H2**	**35**	**32**	**25**	**37**
OX4458-D1	34	37	7	14	OX4458-I1	44	28	3	27
OX4458-D2	33	44	12	15	OX4458-I2	45	24	4	16
OX4458-E1	**46**	**39**	**43**	**41**	OX4458-K	21	20	7	6
OX4458-E2	38	76	43	1	OX4458-L	31	34	28	33
OX4458-F1	**0**	**96**	**56**	**4**					
OX4458-F2	19	18	17	21					

Table 6.1 Segregation of phenotypes in G2 generation of OX4458-Ae lines. Wild type versus transgenic and male versus female ratios were assessed to identify lines with single, autosomal insertion exhibiting strong expression of fluorescent marker. Lines marked in bold were selected for continued breeding.

G2 cross	G3 pupae transgenic		wild type		G4 pupae transgenic		wild type	
	♀	♂	♀	♂	♀	♂	♀	♂
OX4458-A1	**10**	**2**	**46**	**58**	**14**	**26**	**9**	**21**
OX4458-B2	7	19	12	28				
OX4458-E1	**23**	**33**	**32**	**45**	**31**	**75**	**38**	**73**
OX4458-F1	0	100	101	1				
OX4458-G1	**29**	**39**	**43**	**45**	**23**	**76**	**11**	**54**
OX4458-H2	**15**	**41**	**47**	**43**	**55**	**98**	**54**	**104**

Table 6.2 Segregation of phenotypes in G3 and G4 generations of OX4458-Ae lines. Wild type versus transgenic and male versus female ratios were assessed to identify lines with single, autosomal insertion and strong expression of fluorescent marker. Lines marked in bold were selected for further experiments. Higher numbers of males in G4 were caused by increased mortality of larvae and pupae due to breeding conditions – probably overcrowded pots. As males are the first to pupate and reach adulthood they produced higher adult survivors' numbers.

6.2.1.2. OX4627

Injections were performed following the same protocol. 805 pre-blastoderm embryos were injected. Post-injection survival was as follows:

805 eggs → 144 larvae → 46 ♀ + 51 ♂ (pupae) → 40 ♀ + 44 ♂ (adults)

Six G0 crosses were set-up with surviving adults – of these 4 produced transformants. Subsequent crosses and line analysis brought the results shown in *Tables 6.3 and 6.4.*

G1 cross	G2 pupae			
	transgenic		wild type	
	♀	♂	♀	♂
OX4627-A1	45	104	53	0
OX4627-A2	45	88	38	1
OX4627-B1	8	11	13	13
OX4627-B2	25	28	26	35
OX4627-C2	0	18	19	1
OX4627-D1	70	1	3	86
OX4627-D2	27	24	40	46

Table 6.3 Segregation of phenotypes in G2 generation of OX4627-Ae lines. Wild type versus transgenic and male versus female ratios were assessed to identify lines with single, autosomal insertion and strong expression of fluorescent marker.

G2 cross	G3 pupae				G4 pupae			
	transgenic		wild type		transgenic		wild type	
	♀	♂	♀	♂	♀	♂	♀	♂
OX4627-A1a	1	24	27	1				
OX4627-A1b	0	36	36	1				
OX4627-B1	**40**	**70**	**45**	**69**	**15**	**57**	**31**	**46**
OX4627-B2	**52**	**55**	**40**	**47**	**16**	**16**	**7**	**22**
OX4627-C2	0	117	117	1				
OX4627-D1	**36**	**44**	**38**	**39**	**51**	**55**	**29**	**95**
OX4627-D2 (♀)	32	34	33	37				
OX4627-D2 (♂)	**63**	**69**	**58**	**59**	**19**	**53**	**25**	**66**

Table 6.4 Segregation of phenotypes in G3 and G4 generations of OX4627-Ae lines. Single, autosomal insertion lines marked in bold, were selected for further experiments.

Only lines marked in bold were kept and used in further experiments. OX4627-C2 samples were, as for OX-4627-F1, kept for insertion site characterisation.

6.2.1.3. OX4635

(Injections of OX4635 transposon and establishment of transgenic lines were done by P. Gray). Injections were performed following the same protocol as before. 2398 pre-blastoderm embryos were injected. Post-injection survival was as follows:

2398 eggs → 43 larvae → 7 ♀ + 16 ♂ (adults)

Surviving adults divided into 5 groups – 2 female and 3 male groups – for G0 crosses. Transformants were found in 3 G1 groups. Based on distinct phenotypes 7 possible independent lines were identified: four in two male lineages or "pools" (P1B, P1C, P1D, and P3) and three in one female lineage (P1G, P1H, and P1J). Many of them did not survive past the G1 stage. Subsequent crosses and line analysis were as shown in *Table 6.5*.

Again, only lines marked in bold were propagated further and used in subsequent experiments. They were selected because they were definitely different insertions – coming from two different original G0 male pools.

G2 larvae		**G4 Cross**	**G5 pupae**			
transgenic	**wild type**		**transgenic**		**wild type**	
			♀	♂	♀	♂
99	**136**	**OX4635-P1-Cib-♂**	**12**	**68**	**72**	**3**
183	179	OX4635-P1-Hiii-♀	19	32	34	2
197	205	OX4635-P3i-♂	16	37	39	35
79	**85**	**OX4635-P3iii-♂**	**26**	**47**	**61**	**58**

Table 6.5 Segregation of phenotypes in G4 and G5 generations of OX4635-Ae lines. Lines marked in bold were selected for further experiments. Wild type versus transgenic and male versus female ratios suggest single, autosomal insertion in case of both P3 lines. Both P1 lines show higher number of transgenic males comparing to transgenic females then would be expected in case of autosomal insertion.

6.2.2. Testing the conditional male sterility in *Aedes aegypti*.

The general scheme for performing male sterility tests – hatch-rate assays – was as described previously.

6.2.2.1. Topi-tTAV-driven expression of Dm-Protamine-FokICD (OX4286-B x OX4458)

Repressible male-specific sterility in *Aedes aegypti* was first tested in double-heterozygotes obtained from crosses between Topi-tTAV expressing OX4286-B line and tetO-DmProtamine-FokI containing OX4458 lines. *Table 6.6* shows performed crosses and numbers of males and females in each phenotype group, while *Figure 6.4* shows combined numbers in each phenotype. OX4286-B expresses an eye-localised fluorescent marker and OX4458 expresses a whole body marker. Fertility of individuals bearing both alleles and reared either in repressible (+tet) or in permissive conditions (-tet) was assayed in hatch-rate tests.

G0		G1 pupae							
Cross	Tet	Eyes + Body		Eyes only		Body only		Wild type	
		♂	♀	♂	♀	♂	♀	♂	♀
OX4458-A1 x OX4286-B	-	24	12	33	15	0	10	2	8
OX4458-E1 x OX4286-B	-	20	7	20	9	1	17	2	11
OX4458-G1 x OX4286-B	-	57	31	35	10	3	9	2	9
OX4458-H2 x OX4286-B	-	11	1	9	1	0	11	0	10
OX4458-A1 x OX4286-B	+	31	12	20	18	1	3	0	9
OX4458-E1 x OX4286-B	+	49	10	34	13	0	21	1	25
OX4458-G1 x OX4286-B	+	45	33	29	19	4	16	2	14
OX4458-H2 x OX4286-B	+	14	3	4	2	0	10	2	9

Table 6.6 OX4286B (3xP3-DsRe eye marker) and OX4458 (hr5IE1-DsRed body marker) crosses and segregation of phenotypes in the resulting progeny. Segregation data suggest that there are several Topi-tTAV insertions in OX4286B line – some of them being linked to the male sex determination locus. Crosses reared in the absence of tetracycline are in the red font, crosses reared in the presence of tetracycline are in the black font.

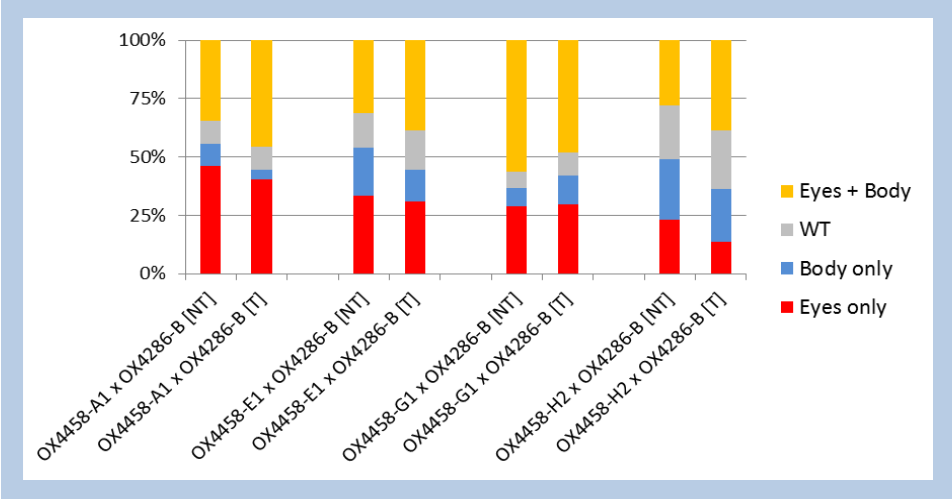


Figure 6.4 Segregation of phenotypes in the progeny of OX4286B and OX4458 crosses. Graph shows the relative abundance of each of the four phenotypes in the progeny of OX4458 x OX4286 crosses. Higher proportion of pupae with fluorescent eye marker (3xP3-DsRed in OX4286) compared to pupae with fluorescent body marker (hr5IE1-DsRed in OX4458) suggests several insertions in OX4286-B line.

Hatch-rate of embryos fathered by males carrying both OX4286B and OX4458 alleles and reared without tetracycline showed no significant difference comparing to "on-tet", wild type and female controls [*Fig. 6.5*].

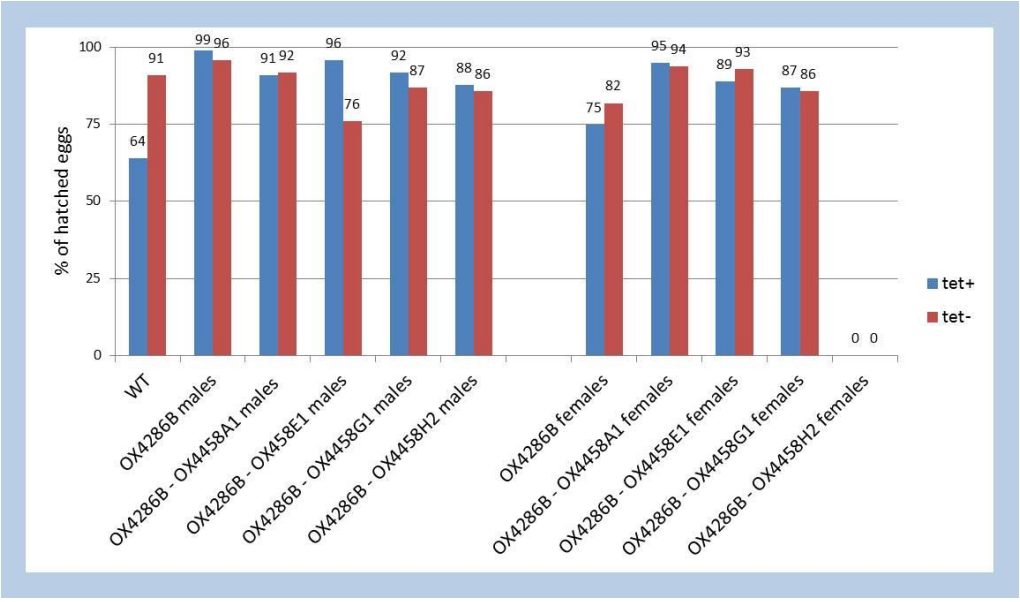


Figure 6.5 Hatch-rate assay of *Aedes aegypti lines* carrying both Topi-tTAV and tetO-Dm-Protamine-FokI alleles. Progeny of crosses between OX4286B line and OX4458 lines were reared either on a diet with (tet+) or without tetracycline (tet-). Males (or females) carrying both driver and effector alleles were crossed to the wild type and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type and OX4286B males' crosses with wild type females were used as controls.

6.2.2.2. Topi-tTAV-driven expression of Ae-Protamine-FokICD (OX4286-B x OX4627)

After unsuccessful attempts to achieve male sterility utilising *Drosophila melanogaster* protamine, crosses between OX4286-B line and OX4627 effector lines containing tetO-AeProtamine-FokICD were performed. As endogenous *Aedes aegypti* protamine was already shown to work in fusion with FokI nuclease in a constitutive sterility system and hence is very unlikely to malfunction, such crosses are better suited to test tTAV under the control of Topi promoter as the driver for the nuclease. Progeny of crosses between OX4286-B line and four OX4627 lines (B1, B2, D1 and D2) were reared in the presence or absence of tetracycline and scored for different phenotypes as shown in the *Table 6.7* and in *Figure 6.6*.

G0 cross		G1 pupae							
	Tet	Eyes + Body		Eyes only		Body only		Wild type	
		♂	♀	♂	♀	♂	♀	♂	♀
OX627-B1 x OX4286-B	-	69	38	81	52	1	18	2	14
OX4627-B2 x OX4286-B	-	52	19	60	31	3	20	4	15
OX4627-D1 x OX4286-B	-	29	17	37	12	0	21	1	20
OX4627-D2 x OX4286-B	-	32	26	38	27	1	20	0	20
OX627-B1 x OX4286-B	+	47	35	38	37	0	10	1	16
OX4627-B2 x OX4286-B	+	67	29	68	25	0	27	2	26
OX4627-D1 x OX4286-B	+	8	9	5	9	0	1	0	6
OX4627-D2 x OX4286-B	+	39	20	32	17	0	17	0	14

Table 6.7 OX4286B (3xP3-DsRed eye marker) and OX4627 (hr5IE1-DsRed body marker) crosses and segregation of phenotypes in the resulting progeny. The data suggest that there are several Topi-tTAV insertions in OX4286B line – some linked to the male sex determination locus. Crosses reared in the absence of tetracycline are in the red font, crosses reared in the presence of tetracycline are in the black font.

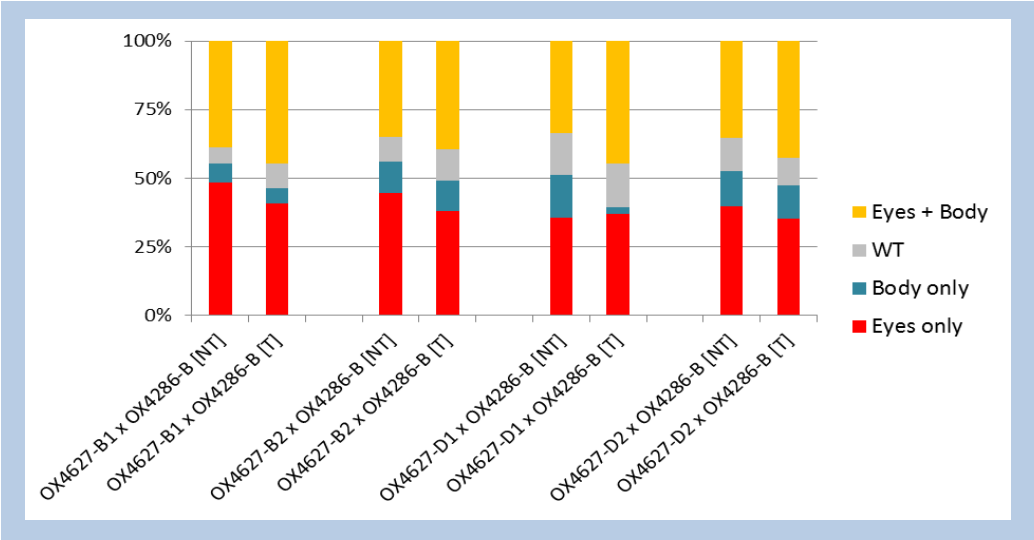


Figure 6.6 Segregation of phenotypes in progeny of OX4286B and OX4627 crosses. Higher proportion of pupae with fluorescent eye marker (3xP3-DsRed in OX4286) compared to pupae with fluorescent body marker (hr5IE-DsRed in OX4627) suggests several insertions in OX4286-B line.

Double-heterozygous males were crossed to wild type females and resulting embryos scored for hatch rate. Wild type, OX4627 only, and female controls were included. Significant (up to 100%) reduction of embryonic hatch rate was observed in all 4 samples of males carrying both alleles reared on a diet without tetracycline [*Fig. 6.7*].

Hatch-rates in OX4286B and female controls were unaffected – confirming the sex-specificity of nuclease expression – apart from a noticeable drop in the numbers of hatched eggs in case of OX4286B – OX4627D1 female sample.

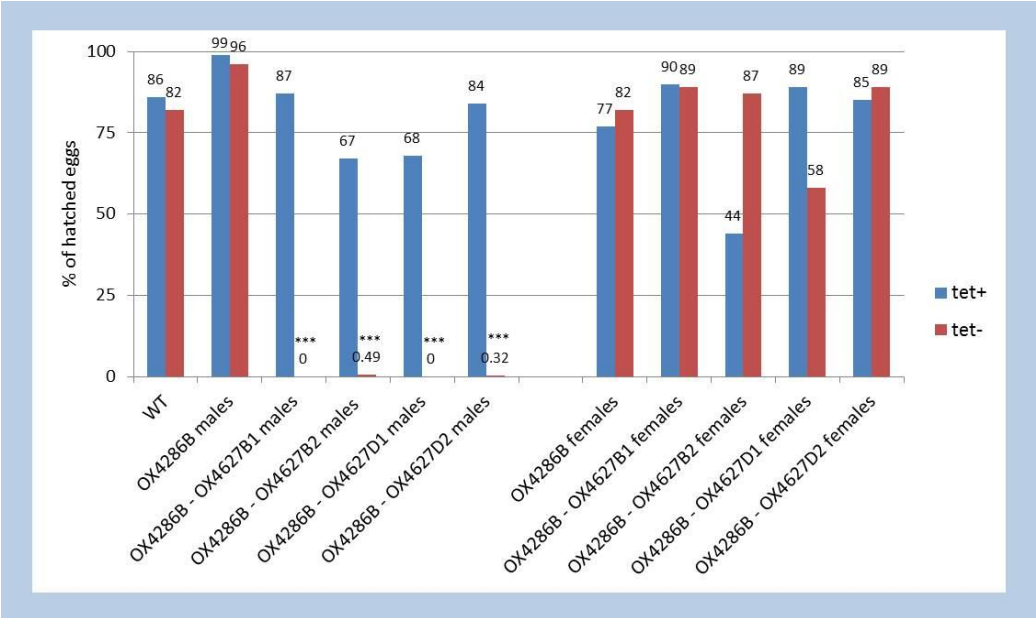


Figure 6.7 Hatch-rate assay of *Aedes aegypti lines* carrying both Topi-tTAV and tetO-Ae-Protamine-FokI alleles. Progeny of crosses between OX4286B line and OX4627 lines were reared either on a diet with (tet+) or without tetracycline (tet-). Males (or females) carrying both driver and effector alleles were crossed to the wild type and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type and OX4286B males' crosses with wild type females were used as controls. Crosses where highly significant male sterility was observed (chi-squared test, P<0.0001) are marked with asterisks.

6.2.2.3. β2-tub-tTAV-driven expression of Dm-Protamine-FokICD (OX4635 x OX4458)

To test the suitability of the β2-tubulin promoter from *Aedes aegypti* for use in our conditional male-sterility system, the same crosses with two effector lines were carried out as in case of testing Topi promoter. First, crosses between OX4635 (β2-tub-tTAV) line and OX4458 (tetO-DmProt-FokICD) were carried out. Two OX4635 lines (P1-Cib and P3iii) and four OX4458 lines (A1, E1, G1, and H2) were tested. Progeny of these crosses were reared in the presence or absence of tetracycline and scored for different phenotypes as shown in *Table 6.8* and in *Figure 6.8*. OX4635 expresses AmCyan as fluorescent marker while OX4458 expresses DsRed.

G0		G1 pupae							
Cross	Tet	Cyan + Red		Cyan only		Red only		Wild type	
		♂	♀	♂	♀	♂	♀	♂	♀
OX4458-A1 x OX4635-P1Cib	-	4	0	60	5	5	37	4	46
OX4458-E1 x OX4635-P1Cib	-	18	1	26	5	8	40	4	43
OX4458-G1 x OX4635-P1Cib	-	43	4	46	4	13	41	4	47
OX4458-H1 x OX4635-P1Cib	-	32	2	54	3	1	50	7	47
OX4458-A1 x OX4635-P3iii	-	0	0	28	25	20	17	64	56
OX4458-E1 x OX4635-P3iii	-	12	1	24	19	44	37	48	52
OX4458-G1 x OX4635-P3iii	-	4	5	13	12	40	30	24	31
OX4458-H1 x OX4635-P3iii	-	10	6	10	11	8	15	18	12
OX4458-A1 x OX4635-P1Cib	+	11	0	34	2	2	26	1	28
OX4458-E1 x OX4635-P1Cib	+	4	0	16	0	3	8	4	12
OX4458-G1 x OX4635-P1Cib	+	3	1	8	0	0	14	0	20
OX4458-H1 x OX4635-P1Cib	+	9	2	18	0	0	23	3	21
OX4458-A1 x OX4635-P3iii	+	3	3	16	9	17	13	11	18
OX4458-E1 x OX4635-P3iii	+	5	3	10	12	10	8	7	19
OX4458-G1 x OX4635-P3iii	+	7	1	3	3	5	11	7	8
OX4458-H1 x OX4635-P3iii	+	3	4	4	1	12	1	6	7

Table 6.8 OX4635 (AmCyan) and OX4458 (DsRed) crosses and segregation of phenotypes in the resulting progeny. Observed phenotype segregation is consistent with previous observation that all OX4458 lines and OX4635-P3iii line contained single, autosomal insertions of transgenes, while OX4635-P1Cib line showed segregation of phenotype suggesting sex linkage. Crosses reared in the absence of tetracycline are in the red font, crosses reared in the presence of tetracycline are in the black font.

Double-heterozygous males were crossed to wild type females and resulting embryos scored for hatch-rates.

This time only wild type controls were included – no transgenic female control crosses were performed. Some reduction of hatch-rate was observed in the case of OX4635-P1Cib – OX4458-A1 sample in the absence of tetracycline [*Fig.6.9*]. Unfortunately, there is lack of data on OX4458-A1 allele in combination with the second β2-tubulin-tTAV allele (P3iii), as we did not obtain double heterozygotes of this genotype. Reduction of hatch-rate was also noticed in embryos fathered by

OX4635-P3iii – OX4458-G1 males (P1Cib – G1 male fertility level seems similar to wild type).

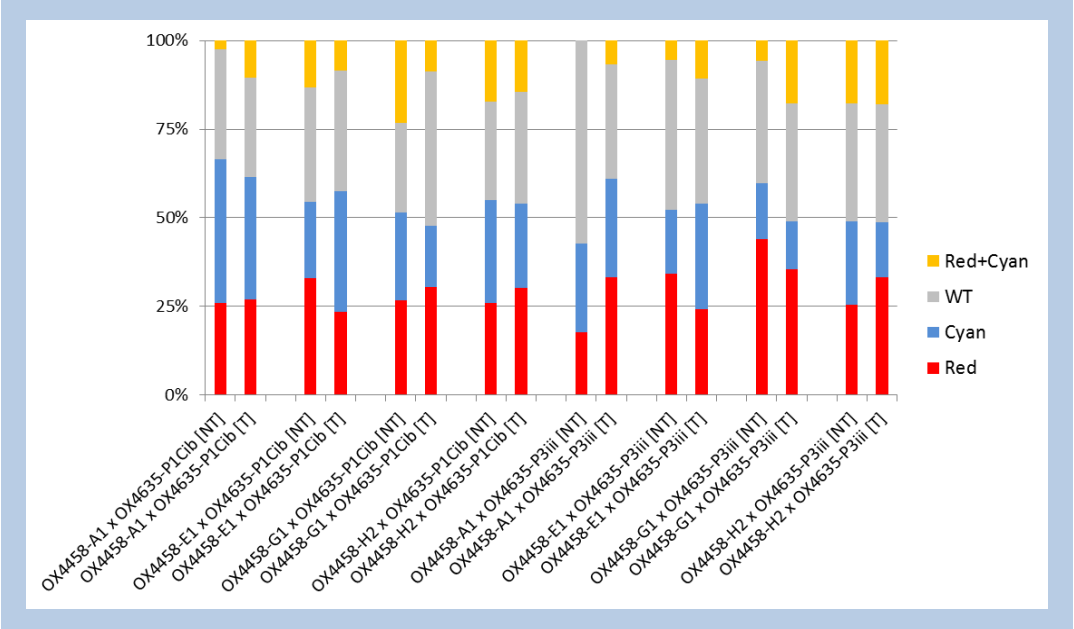


Figure 6.8 Segregation of phenotypes in progeny of OX4635 and OX4458 crosses.

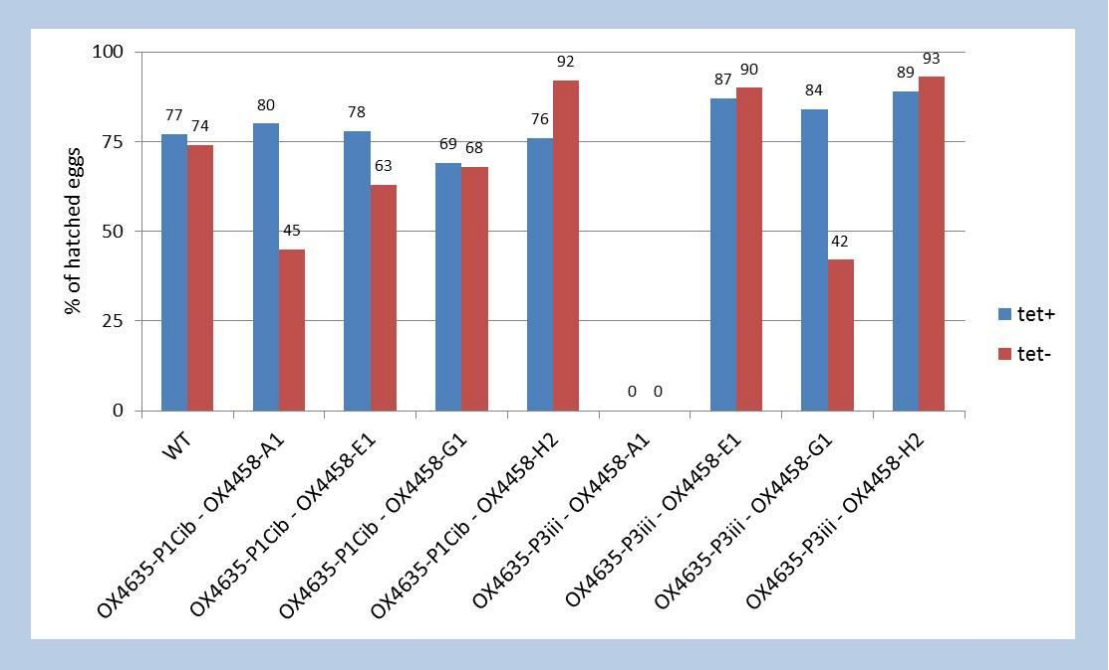


Figure 6.9 Hatch-rate assay of *Aedes aegypti lines* carrying both β2-tubulin-tTAV and tetO-Dm-Protamine-FokI alleles. Progeny of crosses between OX4635 and OX4458 lines were reared either on a diet with (tet+) or without tetracycline (tet-). Males carrying both driver and effector alleles were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type males' crosses with wild type females were used as controls.

6.2.2.4. β2-tub-tTAV-driven expression of Ae-Protamine-FokICD (OX4635 x OX4627)

Lastly, the combination of β2-tubulin driven tTAV and tetO-Ae-Protamine-FokICD was tested. Again, two OX4635 lines (P1-Cib and P3iii) and four OX4627 lines (B1, B2, D1, and D2) were tested. Progeny of these crosses were reared in the presence or absence of tetracycline and scored for different phenotypes as shown below in the *Table 6.9* and in *Figure 6.10*. AmCyan fluorescence indicates presence of OX4635 allele and DsRed fluorescence presence of OX4627 allele.

G0 cross	Tet	G1 pupae							
		Cyan + Red		Cyan only		Red only		Wild type	
		♂	♀	♂	♀	♂	♀	♂	♀
OX627-B1 x OX4635-P1Cib	-	12	**9**	17	16	18	17	19	21
OX4627-B2 x OX4635-P1Cib	-	18	12	24	30	43	41	44	42
OX4627-D1 x OX4635-P1Cib	-	32	2	27	3	0	34	6	46
OX4627-D2 x OX4635-P1Cib	-	8	0	17	1	0	17	0	15
OX4627-B1 x OX4635-P3iii	-	26	8	31	14	48	36	33	42
OX4627-B2 x OX4635-P3iii	-	5	5	20	15	16	19	13	22
OX4627-D1 x OX4635-P3iii	-	24	23	28	33	47	31	44	39
OX4627-D2 x OX4635-P3iii	-	14	21	29	13	26	16	31	27
OX627-B1 x OX4635-P1Cib	+	4	3	3	1	6	7	2	4
OX4627-B2 x OX4635-P1Cib	+	5	9	9	18	16	18	18	10
OX4627-D1 x OX4635-P1Cib	+	11	0	10	0	0	17	1	9
OX4627-D2 x OX4635-P1Cib	+	2	0	2	0	0	2	0	3
OX4627-B1 x OX4635-P3iii	+	10	11	10	10	9	11	12	11
OX4627-B2 x OX4635-P3iii	+	5	4	6	3	11	5	6	8
OX4627-D1 x OX4635-P3iii	+	9	6	16	6	16	19	12	16
OX4627-D2 x OX4635-P3iii	+	14	5	9	6	20	16	22	26

Table 6.9 OX4635 and OX4627 crosses and segregation of phenotypes in the resulting progeny.

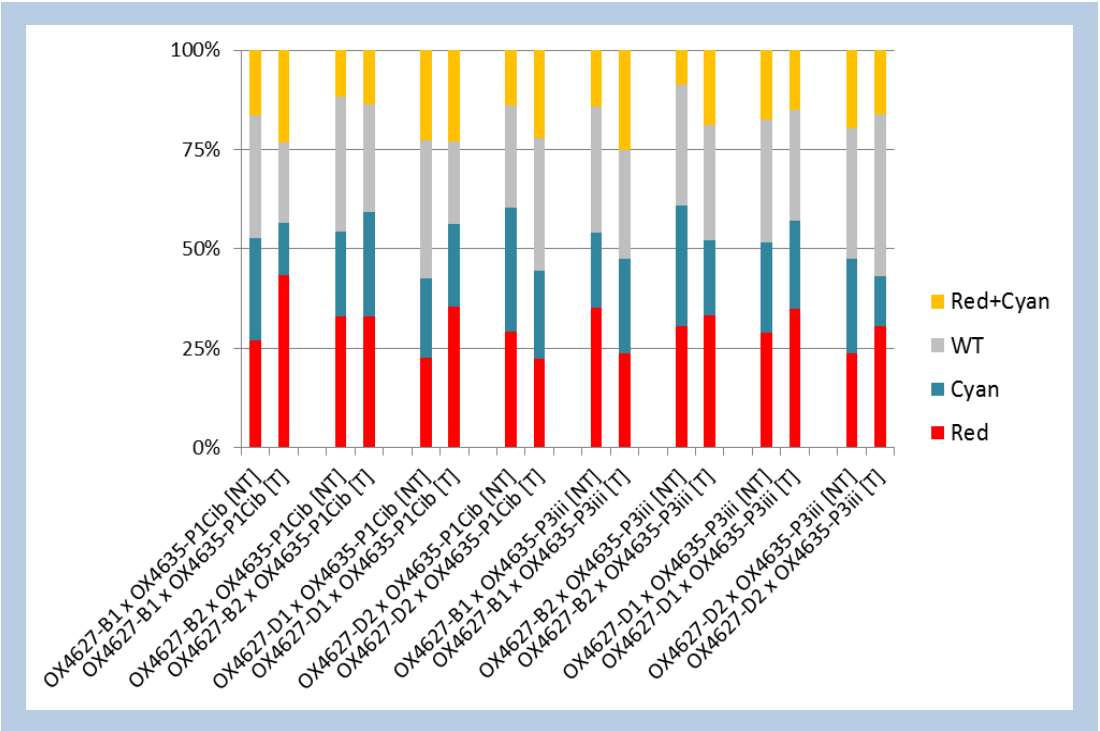


Figure 6.10 Segregation of phenotypes in progeny of OX4635 and OX4627 crosses.

Double-heterozygous males carrying both β2-tubulin-tTAV (OX4635) and tetO-Ae-Protamine-FokICD (OX4627) alleles were crossed to wild type females and resulting embryos scored for hatch rate [*Fig. 6.11*]. Wild type controls were included but no transgenic female control crosses were performed. OX4627-B1 and B2 alleles seemed to cause quite strong sterility (reduction in embryos hatch rate) in combination with both OX4635 alleles, although P1Cib – B2 and P3iii – B3 allele combinations showed better repressibility of the phenotype in the presence of tetracycline.

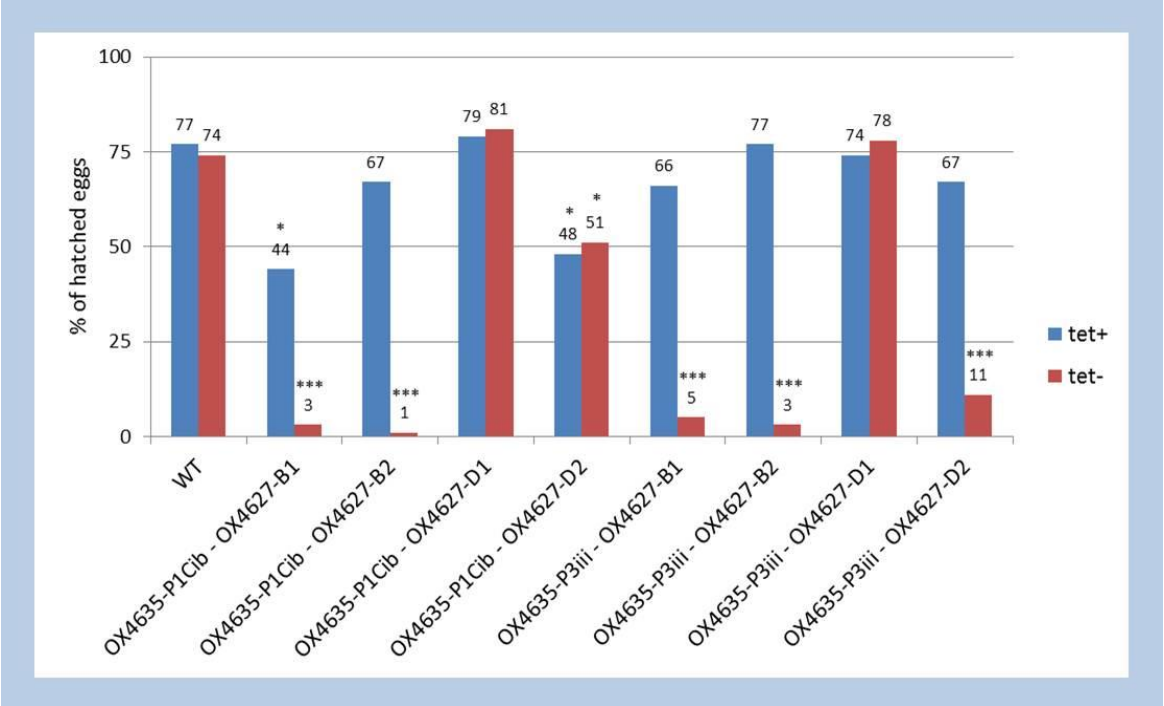


Figure 6.11 Hatch-rate assay of *Aedes aegypti* lines carrying both β2-tubulin-tTAV and tetO-Ae-Protamine-FokI alleles. Progeny of crosses between OX4635 and OX4627 lines was reared either on a diet with (tet+) or without tetracycline (tet-). Males carrying both driver and effector alleles were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type males' crosses with wild type females were used as controls. Crosses where highly significant male sterility was observed (chi-squared test) are marked with asterisks.

6.3. Conclusions

The above results show that OX4627 effector lines (with *Aedes aegypti* protamine fused with FokICD) performed much better than OX4458 lines (with *Drosophila melanogaster* protamine instead) in crosses with both Ae-Topi-tTAV and Ae-β2-tubulin-tTAV promoter lines.

It is not certain why *Drosophila melanogaster* protamine did not work in *Aedes aegypti* and whether this is the result of inhibition of transcription, translation, or binding to DNA of these protamines.

As both protamines come from different organisms, it could be possible that one obtained from *Drosophila melanogaster* contains codons that are very rare in *Aedes aegypti*, hence causing translational delays or even ribosome stalling resulting in little or no protein being produced. However, examination of available data on codon usage frequencies reveals only minor differences between these two organisms, as shown in *Figure 6.12*. In particular, the two codons with quite high frequency difference – UUA and ACA – occur, respectively, only once and not at all in *Drosophila melanogaster* protamine sequence. This makes translational inhibition seem unlikely to be the principal reason for the lack of function of the Dm-protamine-based construct.

The common feature of protamines from different organisms is the presence of arginine repeat motifs. This high arginine content, ranging typically from 60% - 80%, makes protamines strongly basic proteins, which enables efficient DNA binding [4, 60, 98].

UUU 11.8/15.4	UCU 8.8/10.7	UAU 11.2/18.9	UGU 8.8/7.3
UUC 30.5/24.0	UCC 15.7/12.1	UAC 23.7/18.5	UGC 12.3/8.9
UUA 5.0/12.9	UCA 8.8/13.8	**UAA** 1.1/2.3	**UGA** 0.6/0.5
UUG 18.9/26.8	UCG 18.5/13.4	**UAG** 0.6/0.4	UGG 11.6/9.0
CUU 9.5/9.2	CCU 8.6/12.7	CAU 11.0/12.5	CGU 11.0/15.5
CUC 11.5/11.6	CCC 10.7/13.0	CAC 15.3/8.0	CGC 10.1/10.9
CUA 7.7/6.8	CCA 15.4/21.8	CAA 17.2/25.3	CGA 9.5/4.6
CUG 32.2/12.1	CCG 16.8/8.1	CAG 25.3/16.6	CGG 8.5/3.6
AUU 17.5/25.9	ACU 10.9/13.0	AAU 19.9/31.6	AGU 12.2/12.2
AUC 27.9/19.1	ACC 20.2/15.4	AAC 30.4/23.3	AGC 14.5/14.1
AUA 7.1/12.1	ACA 9.6/15.8	AAA 23.2/30.3	AGA 5.1/5.4
AUG 23.7/22.8	ACG 13.7/7.2	AAG 35.2/26.9	AGG 4.3/2.2
GUU 17.2/20.5	GCU 19.1/34.7	GAU 31.7/28.0	GGU 17.8/37.2
GUC 17.8/12.8	GCC 26.1/25.0	GAC 25.1/15.8	GGC 16.8/25.8
GUA 9.9/13.0	GCA 13.2/22.4	GAA 34.0/30.8	GGA 24.1/9.7
GUG 20.7/19.2	GCG 12.2/13.6	GAG 24.9/24.5	GGG 6.0/2.9

Figure 6.12 Codon usage frequencies in *Aedes aegypti* (blue) and *Ceratitis capitata* (red). Table shows how frequently, on average, each codon occurs in a 1000 triplet long protein coding DNA sequence of each organism. Start and Stop codons are shown in bold. Some codons with lower frequencies in *Aedes aegypti* are underlined. *Data obtained from Codon Usage Database from Kazusa DNA Research Institute website (http://www.kazusa.or.jp/codon/).*

Apart from arginine-rich motifs there seems to be little conservation at the protein sequence level – as alignments of *Drosophila melanogaster* and *Aedes aegypti* protamines from OX4458 and OX4627 constructs show [*Fig. 6.13*]. As such, high arginine content seems to be the most important feature of protamines for their correct function. Taken together with lack of sequence specificity in the DNA binding by protamines, it is hard to imagine why an arginine-rich protamine from one organism would not be able to bind to DNA of another organism. One possibility would be mediation of DNA binding by other proteins involving protein-protein interaction. It is theoretically possible considering the replacement of histones by transition proteins (TP) then finally by protamines [60]. In such a scenario, protamines binding to DNA could be facilitated by binding to TPs, and

this interaction could be directed by specific protein sequence / structures. *Drosophila melanogaster* derived protamine could, in such case, fail to bind to *Aedes aegypti* transition proteins, explaining the lack of function of the Dm-protamine-based nuclease.

Figure 6.13 Alignment of protein sequences of protamines from *Drosophila melanogaster* and *Aedes aegypti* used in OX4458 and OX4627 constructs. Lower panel shows the legend explaining colour- and mark- coding used. *The alignment was performed using Clustal Omega on-line tool from the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/clustalo/)*

The OX4458 construct contains the whole *Drosophila melanogaster* protamine gene, with two introns, while the OX4627 one contains the coding sequence of *Aedes aegypti* protamine. Yet another possibility is, then, inability of correct splicing of fruit fly protamine by mosquito's splicing machinery, but this is also only speculation as no RT-PCR were performed.

Co-expression with protamine-FokI nuclease, as a protein fusion, of a fluorescent marker would be of great help in elucidating reasons for the lack of expected

phenotype in OX4458 lines crossed with tTAV trans activator source. Combined with RT-PCR, this would allow testing at which stage of the expression of protamine the problem is occurring. For reasons mentioned before, for fusion with protamine-FokICD a monomeric fluorescent protein would have to be used, and none was readily available at the time of making of both OX4458 and OX4627 constructs.

A stronger sterilisation effect was seen in crosses where nuclease expression was driven by the Topi promoter, compared to β2-tubulin. One potential reason could be removal of any, or both of B2UE1 and B2UE2 motifs, shown in *Figure 5.2.* Both of them have been indicated in establishing tissue-specificity of β2-tubulin expression, especially B2UE1, which has been shown to direct sperm-specific expression when placed in heterologous promoters [83, 105].

Removal of B2UE1 with β2-tubulin 5'UTR might have affected the strength and tissue specificity of the promoter (although keeping it could revert timing of the expression to its original state – which renders β2-tubulin promoter unsuitable for bipartite expression system, as was shown in the case of the *Ceratitis capitata* homologue).

The stronger effect exerted by the Topi promoter could also be related to more than one insertion of Topi-tTAV allele. As indicated by the phenotype segregation data, there are several Topi-tTAV insertions in OX4286B line – some of them being linked to the male sex determination locus. *Aedes aegypti* lack sexually dimorphic chromosomes, instead sex is determined by the presence or absence of male sex-determination locus (M) [21, 97]. These multiple copies could explain strong sterile phenotype penetrance.

In summary, in the course of experiments described in this chapter we were able to successfully identify elements necessary for designing complete, conditional male-specific sterility inducing system in *Aedes aegypti*.

Chapter 7 – A sex-specific transformation marker for *Ceratitis capitata*.

7.1. Introduction

In most sexually reproducing species the ratio of males to females is approximately 1:1; the reason for which has been argued in Fisher's Principle [46]. Unfortunately, in the context of insect rearing, a 1:1 male-to-female ratio is not necessarily optimal. Female reproductive rate is limited by egg production while for males the limiting factor is usually the number of secured matings and not the number of produced sperm [10]. Hence a single male can produce more progeny then a single female, *via* mating with multiple females. Because of that, equal numbers of both sexes in a rearing cage can mean higher than optimal – from the perspective of large-scale insect rearing – input of resources for the production of a given number of eggs. Changing ratios in favour of females (e.g. 1 male per 4 females while keeping numbers of females unchanged) can therefore help in obtaining the same number of eggs from less food and space in the mass production facility. This effect is negligible in small-scale laboratory conditions but could be of substantial benefit in the context of mass production.

To change the male to female ratio a method of distinguishing and separating both sexes is needed that could be applied to early stages of the life cycle – to benefit as much as possible from redirecting resources. This was the one of the reasons behind an attempt to build the sex-specific marker system in the Mediterranean

fruit fly. If successful it could be implemented in the *Ceratitis capitata* male-sterile lines prepared for mass rearing and field use. In such a system males and females would differentially express two fluorescent transformation markers enabling recognition and separation of males from females during either larval or pupal stages. Apart from allowing the selection of female-biased populations for egg production sex-specific marker could also form the basis of sex-sorting system for obtaining pure male populations for release.

At the core of our proposed system lies the differential, sex-specific splicing involved in the sex-determination pathway.

In *Drosophila melanogaster*, the best studied insect sex-determination system, the decision regarding which sex an embryo should develop is performed autonomously in each cell. In other words each cell independently establishes its male or female identity. The primary signal for that is the relative number of sex chromosomes to autosomes – X chromosomes to autosomes to be precise. If the ratio of X:A is 1 (as in XX:AA females) each cell switches on the female developmental pathway; if the ratio is 0.5 (as in XY:AA males) the cell follows the male-type pathway. "Reading" of the ratio is performed by, so called, numerator and denominator proteins (encoded on X chromosomes and autosomes respectively) – transcription factors which compete for the binding of the promoter of the key gene in the sex determination pathway – *Sex-lethal* (*Sxl*).

In females, numerators outcompete denominators and turn on expression of a female-specific splice-variant of *Sex-lethal*. This female-specific version of *sxl* starts the cascade of female-specific splicing of first *transformer (tra)* and then *double-sex (dsx)* gene products – the latter one being a double-switch protein driving either

male or female downstream sex-determining genes, depending on which variant is expressed.

In males, on the other hand, blocking expression of the female specific variant of *Sxl* by denominators leads to expression of "default" male splice-variants of both *Sxl* and *tra* which produce truncated, non-functional proteins. This leads to expression of default, male variant of doublesex [106, 109].

In members of the *Tephritidae*, such as Mediterranean fruit fly, entering a specific sex-determination pathway is not triggered by measuring the ratio of X chromosomes and autosomes as in Drosophilids. Instead, maternally inherited transformer protein initiates female-specific splicing of zygotically expressed *Cctra*, which is later maintained in an auto regulatory fashion. The female specific version of transformer in turn drives female-specific splicing of doublesex mRNA producing a female variant of this protein, responsible for development of female phenotype.

In male tephritids, a Y-linked male-determining factor (M) represses the auto regulatory expression of female transformer variant causing production of inactive, truncated, male variants of Cctra. This ultimately leads to expression of default, male-specific version of doublesex and development of male phenotype [91]. Comparison of both drosophilid and tephritid pathways is shown in *Figure 7.1.*

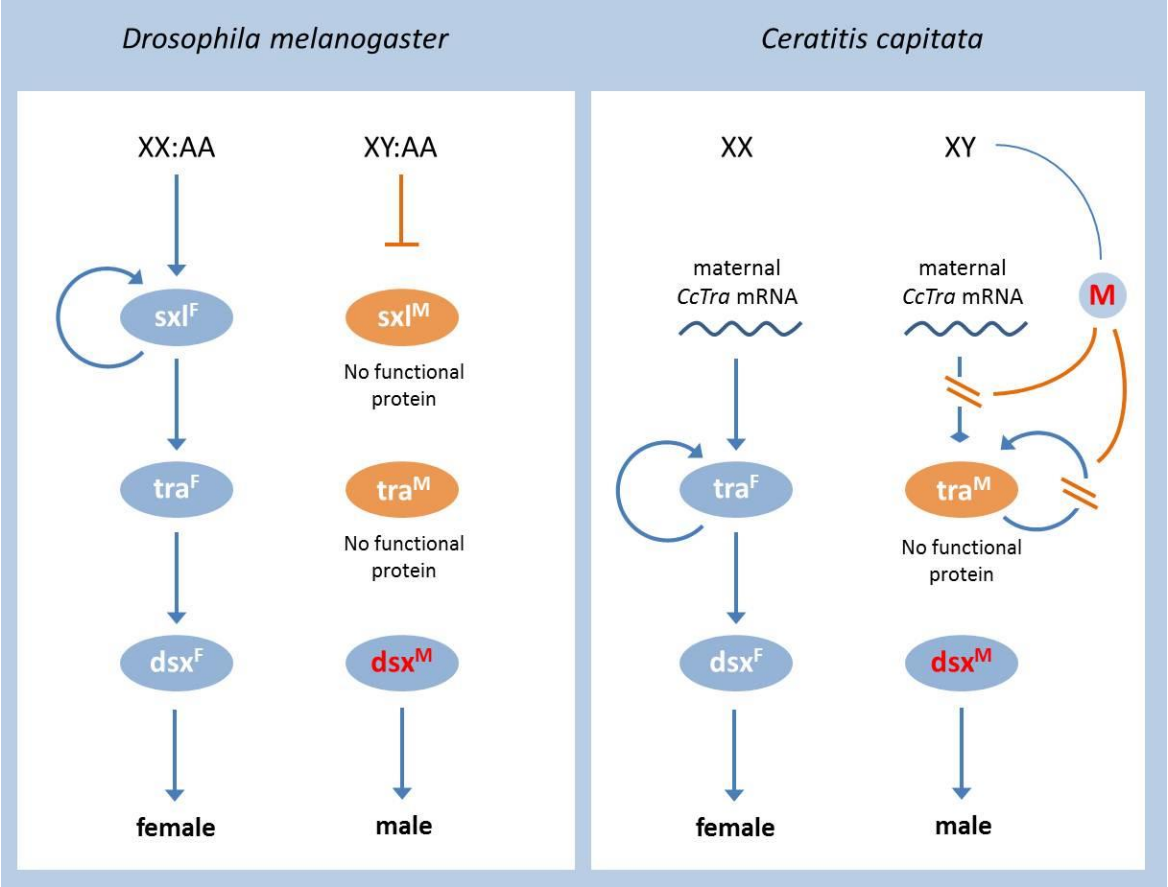


Figure 7.1 Simplified diagrams of sex-determination pathways in *Drosophilae* and *Tephritidae*. Used abbreviations: sxl – sex-lethal, tra – transformer, dsx – doublesex, *Cc* – *Ceratitis capitata*, F – female variant, M – male variant. Adapted from [91] and [106].

Despite being triggered by different initial signals both systems rely on sex-specific alternative splicing. This sex-specific splicing has been previously used in Oxitec to engineer female lethality by expressing in females tetracycline transactivator (tTAV) in a positive feed-back loop. This led to accumulation of tTAV to toxic levels in female tissues resulting in female death. Sex-specificity of this lethal phenotype was achieved by disrupting the tTAV open reading frame with a female-specific intron from the *Ceratitis capitata* transformer gene. Since this intron is spliced out in female mRNA but retained in male mRNA, only females are able to produce functional tTAV [35]. Such a system has a dual function – as a lethal transgene,

killing female progeny that inherits it, and as a sexing mechanism enabling male-only field releases of insects in the SIT or RIDL programs.

For our sex-specific transformation marker we wanted to replicate the mechanism used in this engineered female-lethal allele. Two open reading frames of fluorescent proteins would be separated by the female-specific transformer intron in such a way that in females fusion protein of two fluorescent markers will be produced, while in males retaining of the intron will lead to expression of only the first of the two markers.

Since both alleles (female lethal and sex-specific marker) could be incorporated into *Ceratitis capitata* male-sterile lines prepared for field use, we wanted to avoid the presence of long stretches of identical DNA sequence in the same transposon, which may lead to unwanted recombination events. To achieve this, a sex-specific intron from olive fly (*Bactrocera oleae*) *tra* was used in the sex-specific marker system instead of one from *Ceratitis capitata.* The structure and function of olive fly transformer gene (*BoTra*) and its product was previously characterised by Lagos *et al.* [2007]. They confirmed the alternative splicing of *BoTra*, its maintenance by the auto regulatory mechanism, and the role of the transformer protein in sex-determination in the olive fly. Structures of the olive fly transformer gene and its different splicing variants are shown in *Figure 7.2*.

To drive the expression of sex-specific marker cassette, a muscle actin promoter from Mexican fruit fly *Anastrepha ludens* was chosen. Muscle actin was previously investigated in Oxitec as a candidate for a strong, ubiquitous promoter to drive expression of transformation markers.

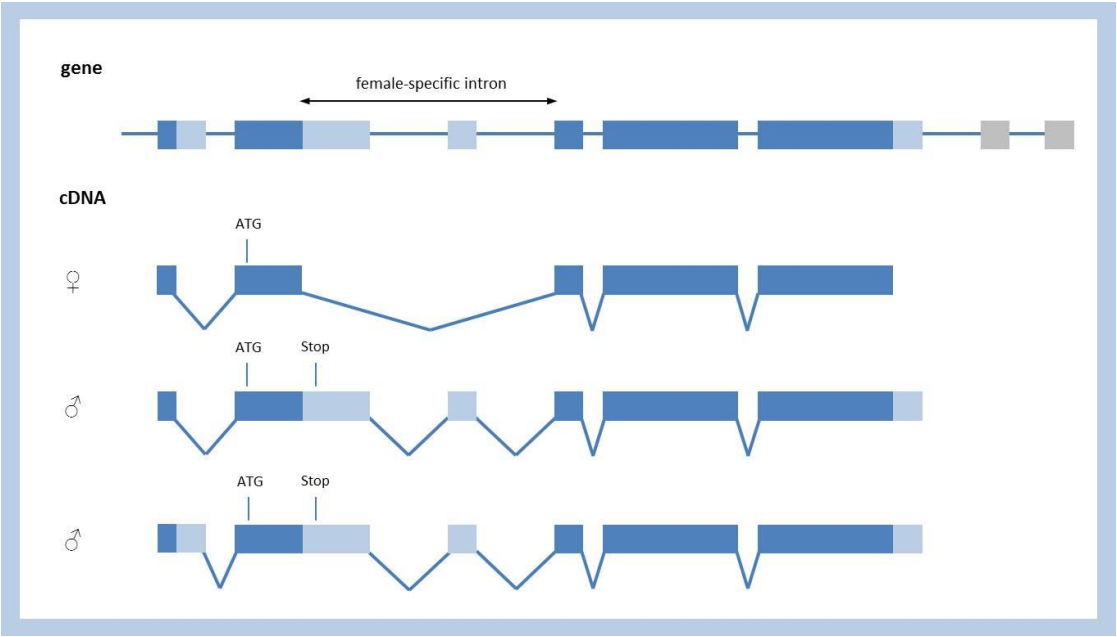


Figure 7.2 *Bactrocera oleae* transformer gene structure and mRNA splice-variants. Exons are depicted as dark blue or light blue boxes, introns as solid lines. The position of the female-specific intron used for a sex-specific transformation marker system is indicated. Adapted from [73].

Based on alignments of known *Drosophila melanogaster* and *Aedes aegypti* sequences, degenerate PCR primers were designed to clone muscle actin from genomes of several insect species – attempts in Mexican fruit fly were the most successful. Promoter, 5'- and 3'UTRs of Mexican fruit fly muscle actin gene were obtained by RACE PCRs and genome walking *(work done by Dr Sarah Scaife*). Ultimately a 2244 bp fragment immediately upstream of translation start was selected as the promoter and its activity was confirmed by driving expression (in a very strong manner) of DsRed in a test construct (*previous Oxitec results*).

Two constructs were designed: OX4628 and OX4676. Both are piggyBac-based transposons and contain a fluorescent marker cassette under the transcriptional control of muscle actin promoter and muscle actin 3'UTR from the Mexican fruit fly.

The fluorescent marker cassette in OX4628 contains the DsRed2 ORF lacking stop codons followed by an olive fly female-specific intron and the ZsGreen ORF without a start codon. Such a design should produce a DsRed-ZsGreen fusion protein in females and DsRed only in males, in which, due to stop codons present in the retained female-specific intron, translation should stop just after DsRed coding sequence. Such permanent fusion of two fluorescent proteins could cause fluorescence quenching problems due to possible Förster resonance energy transfer (FRET) effect.

To eliminate such risk, in OX4676 sequence coding for an ubiquitin tag followed by nuclear localisation signal (nls) was placed upstream of the ZsGreen open reading frame. The ubiquitin tag is intended to direct cleavage of the translated fusion protein, between itself and nuclear localisation signal, separating DsRed and ZsGreen fluorescent domains [124, 125]. Additionally the nls should translocate ZsGreen into the nucleus separating it spatially from (presumably) cytoplasm-localised DsRed, which should help in distinguishing each fluorescent signal. Diagrams of both OX4628 and OX4676, presenting the most important features of design, are shown in *Figure 7.3*.

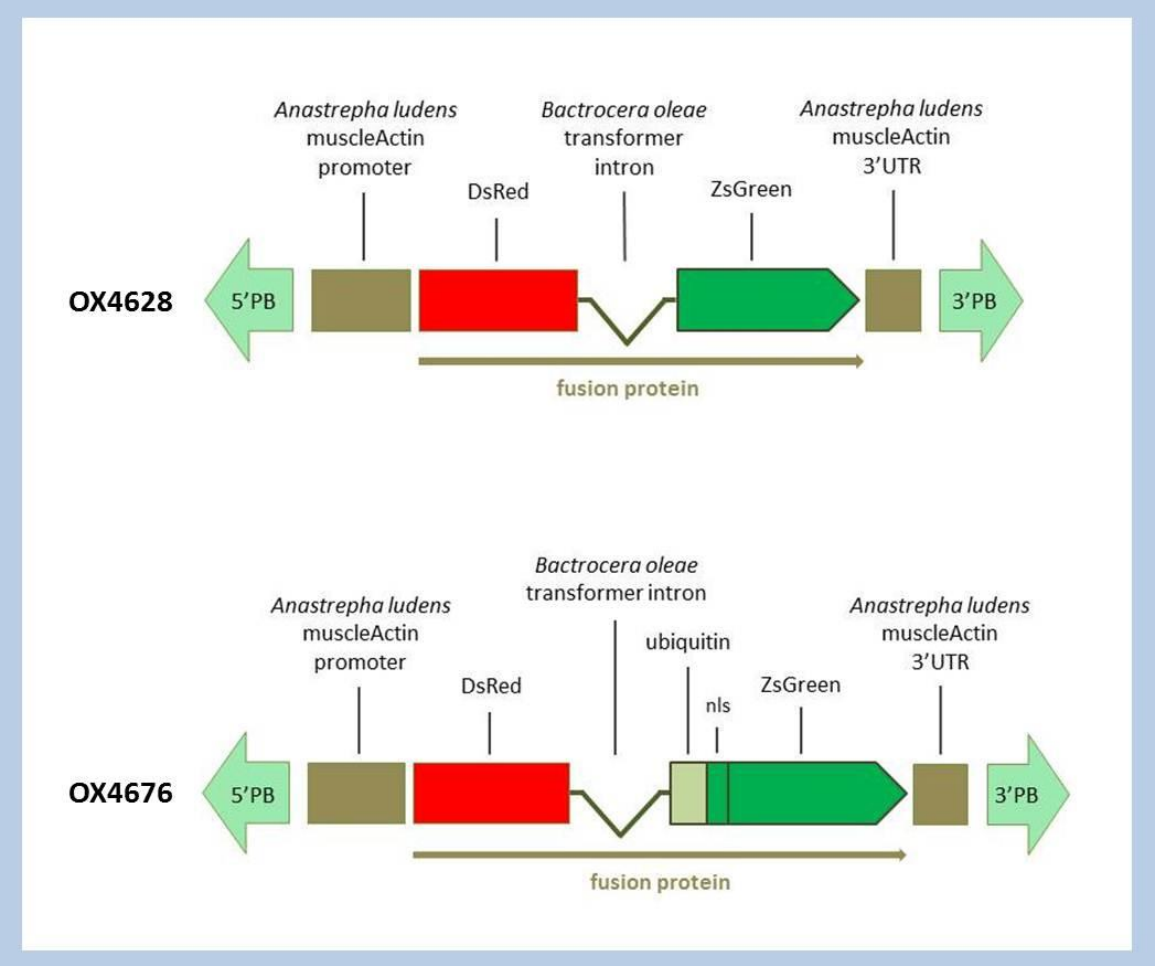


Figure 7.3 Schematic diagrams of OX4628 and OX4676 constructs. Both constructs were designed for sex-specific marking in *Ceratitis capitata*. They contain DsRed-ZsGreen fusions split with female-specific intron from olive fly transformer gene. The variant in OX4676 also contains ubiquitin positioned immediately upstream of ZsGreen for post-translational separation of both fluorescent proteins. Both transposons were supposed to cause different (sex-specific) fluorescent marker expression in males and females.

7.2. Results

7.2.1. Obtaining and analysis of the transgenic OX4628 lines

The OX4628 plasmid was co-injected into pre-blastoderm *Ceratitis capitata* embryos together with piggyBac transposase helper plasmid OX3022 – as

described in Materials and Methods. 1154 embryos were injected. Post-injection survival was as follows:

1154 eggs → 662 larvae → 500 pupae → 224 ♀ + 242 ♂ (adults)

Surviving adults were crossed to wild type in 22 G0 crosses. Transformants were identified by the presence of red fluorescence, since both males and females should express DsRed. Collected pupae were also, just in case, screened for green fluorescence so as not to miss any possible transformants in the "non-red" group. Pupae expressing fluorescent marker were found among progeny of 18 of the G0 crosses – ranging in numbers from 2 to 28 per cross. All collected transformed pupae produced only female adults. To reduce the number of lines, females from only 6 independent groups were crossed (1 female from each group per cross) to wild type males to produce separate insertion lines. Phenotype segregation in resulting G2 pupae and adults is shown in the *Table 7.1.*

	Transgenic		Non-transgenic	
	pupae	adults	pupae	Adults
OX4628-B	17	17 ♀	56	42 ♂ + 9 ♀
OX4628-F	14	13 ♀	27	18 ♂ + 9 ♀
OX4628-M	6	4 ♀	20	14 ♂ + 4 ♀
OX4628-K	12	7 ♀	22	10 ♂ + 5 ♀
OX4628-S	15	13 ♀	11	6 ♂ + 2 ♀
OX4628-W	19	15 ♀	22	16 ♂ + 2 ♀

Table 7.1 Wild type to transgenic and male to female ratios in G_2 OX4628 lines. Only female adults emerged from transgenic pupae. The male versus female ratio was biased towards males in non-transgenic adults. In 4 out of 6 groups there were more non-transgenic flies then transgenic ones.

Again only female adults emerged from transgenic pupae with no males. There were both adult males and females in the non-transgenic group, although the ratio was biased towards males. In 4 out of 6 groups there were more non-transgenic flies then transgenic ones. Unfortunately, as a result of single female G1 crosses, numbers of G2 progeny obtained were too low to draw any definite conclusions. More accurate analysis was performed on the larger, G3 generation.

As shown in *Figure 7.4*, in all OX4628 lines (with the exception of line W) non-transgenic pupae outnumbered transgenic ones approximately 3:1. Transgenic pupae gave rise only to transgenic females, in numbers high enough to exclude the possibility of developmentally arrested males being present among the transgenic pupae.

In the non-transgenic group emerged adults consisting of individuals of both sexes, but approximately twice as many males then females. Total numbers of females; both transgenic and non-transgenic, were similar to the numbers of males. This suggested lack of lethality in any phenotypic or sex group, and we hypothesised that for some reasons inherited transgene has become cryptic in males – half of them possess the transgene but present no phenotype. To test this, 10 randomly chosen males from each OX4628 line were mated with 20 females and progeny scored for fluorescence. Transgenic pupae were indeed found among progeny of all test crosses, although in varying numbers, presumably reflecting different numbers of randomly selected transgenic males in each cross [*Fig. 7.5*].

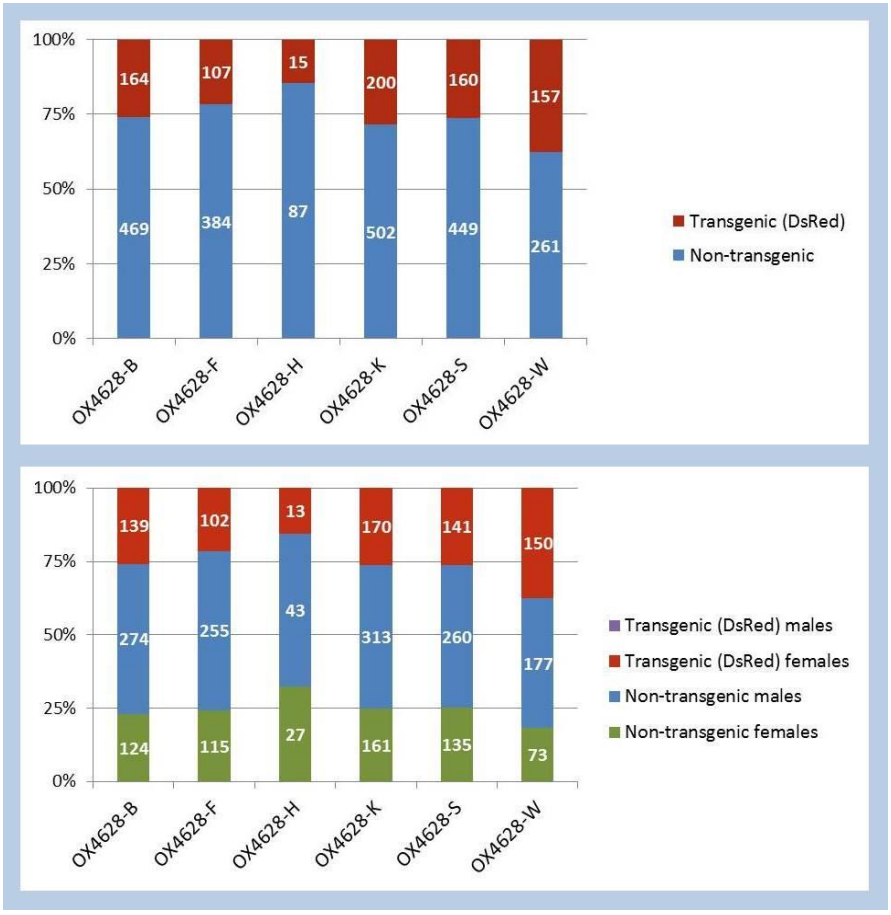


Figure 7.4 Phenotype segregation in OX4628 lines. Upper panel shows ratios of transgenic to non-transgenic pupae. Lower panel shows proportions of ecclosed males and females in both phenotype groups. Actual recorded numbers are shown in the white font on the bars. Results indicate that half of the males probably contain the transgene but it does not produce fluorescent phenotype.

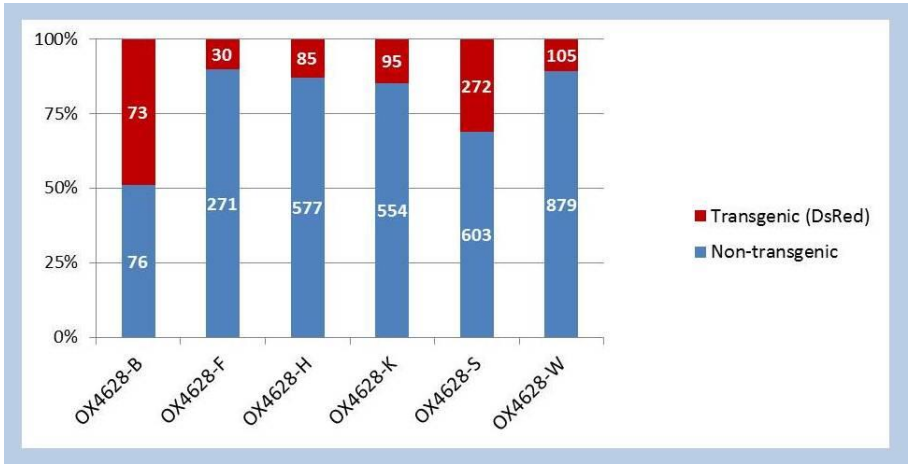


Figure 7.5 Transgenic pupae present among the progeny of non-transgenic OX4628-Cca males. Actual recorded numbers are shown in the white font on the bars. 10 randomly selected males from each OX4628 line were mated with 20 wild type females and progeny was checked for expression of fluorescent marker. Results showed that some of the males contain silent transgene proving that half of the males are transgenic but they do not express the marker.

7.2.2. Obtaining and analysis of the transgenic OX4676 lines

OX4676 plasmid was co-injected into pre-blastoderm *Ceratitis capitata* embryos together with piggyBac transposase helper plasmid OX3022 following the same protocol as for OX4628. 1154 embryos were injected. Post-injection survival was as follows:

1155 eggs → 533 larvae → 325 pupae → 143 ♀ + 164 ♂ (adults)

Surviving GO adults were crossed to wild type in 14 crosses – 8 of these produced transformed flies. Again all of them were females, as shown in *Table 7.2*, as was the case of the OX4628 construct.

	Transgenic	
	pupae	adults
OX4676-E	9	9 ♀
OX4676-G	1	1 ♀
OX4676-I	2	2 ♀
OX4676-J	2	1 ♀
OX4676-K	20	13 ♀
OX4676-K strong	5	3 ♀
OX4676-L	4	3 ♀
OX4676-M	3	0 ♀
OX4676-N	11	8 ♀

Table 7.2 Transformed individuals found in G_1 generation of OX4676 lines. As was the case with OX4628 lines only female adults emerged from transgenic pupae of OX4676 lines as well with no males emerging at all.

Four of these lines were propagated further (as duplicates of single female crosses for each line); the *Table 7.3* below shows phenotype segregations in resulting G2 generation:

	Transgenic		Non-transgenic	
	Pupae	adults	pupae	adults
OX4676-E1	5	none	24	12 ♂ + 6 ♀
OX4676-E2	17**s** +16**w**	(15 ♀)**s** + (4 ♂ + 11 ♀)**w**	46	32 ♂ + 9 ♀
OX4676-K1	6**s** + 14**m** + 13**w**	(3 ♀)**s** + (8 ♀)**m** + (9 ♂ + 3 ♀)**w**	18	11 ♂ + 4 ♀
OX4676-K2	9**s** + 10**w**	(5 ♀)**s** + (5 ♂ + 1 ♀)**w**	17	11 ♂
OX4676-L1	18**s** + 14**w**	(7 ♀)**s** + (9 ♂ + 2 ♀)**w**	28	12 ♂ + 5 ♀
OX4676-N1	23	1 ♂ + 12 ♀	73	39 ♂ + 18 ♀
OX4676-N2	9	none	53	27 ♂ + 15 ♀
	(w – weak, m – medium, s – strong fluorescence)			

Table 7.3 Wild type to transgenic and male to female ratios in G_2 OX4676 lines. There are approximately twice as many non-transgenic males then females among the emerging adults – similarly to OX4628 lines. In 4 out of 7 crosses (E1, E2, N1 and N2) there are substantially more non-transgenic then transgenic pupae. These results suggest similar pattern as in case of OX4628 lines. Especially that it is not certain if weak fluorescent phenotype represents true transgene fluorescence and not auto-fluorescence.

Numbers in the OX4676 G2 generation were low, but still some trends were noticeable. Adults emerging from non-transgenic pupae contained around twice as many males as females. Transgenic groups, in four cases, contained similar numbers of pupae as non-transgenic ones; although classification of "weak transgenics" was ambiguous – these pupae were brighter than ones classified as non-transgenic but it was impossible to identify similar expression patterns as in the case of "strong fluorescent" ones. Noticeably more males than females among adults eclosed from "weak fluorescence" pupae suggests that indeed these might not have been true transgenics, although the numbers are too low to be certain.

It is also worth noting that introduction of an ubiquitin tag, and adding nuclear localisation signal to ZsGreen protein, improved visibility of green fluorescence.

All 7 lines were propagated further by crossing transgenic females from "strong expression" groups to wild type males. Unfortunately, due to independent problems with rearing, only two of these lines survived: OX4676-E2 and OX4676-L1. Nevertheless, these two lines show a similar phenotype and sex segregation as was observed in the case of OX4628 lines [*Fig. 7.6*]. Similarly, randomly selected non-transgenic males produced transgenic progeny in crosses with wild type females.

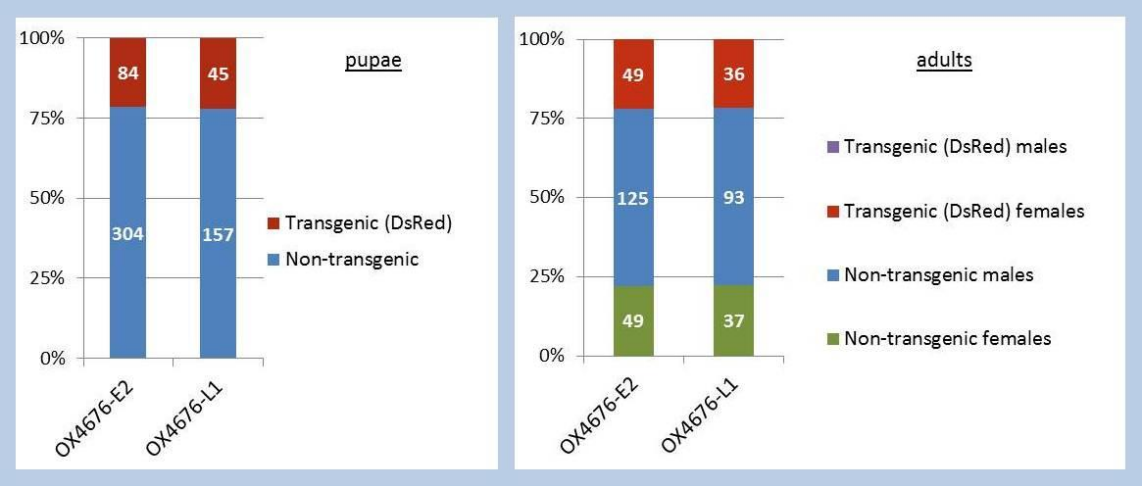


Figure 7.6 Phenotype segregation in OX4676 lines E2 and L1. Left panel shows ratios of transgenic to non-transgenic pupae. Right panel shows proportions of eclosed males and females in both phenotype groups. Actual recorded numbers are shown in the white font on the bars. There are approximately 3 times more non-transgenic than transgenic individuals among the progeny resulting from crosses of females from OX4627 lines E2 and L1 to wild type males. Also, no transgenic males are among the progeny. However, the total number of males is similar to the total numbers of females. These results suggest that half of the males contain the transgene, although it does not express the phenotype.

7.3. Conclusions

It is not certain why our attempts to create sex-specific a transformation marker system were not successful. Although female-specific splicing has apparently

occurred as intended, joining the DsRed and ZsGreen open reading frames and producing visible red and green fluorescence in females [*Fig. 7.7*], the system failed to produce fluorescent males.

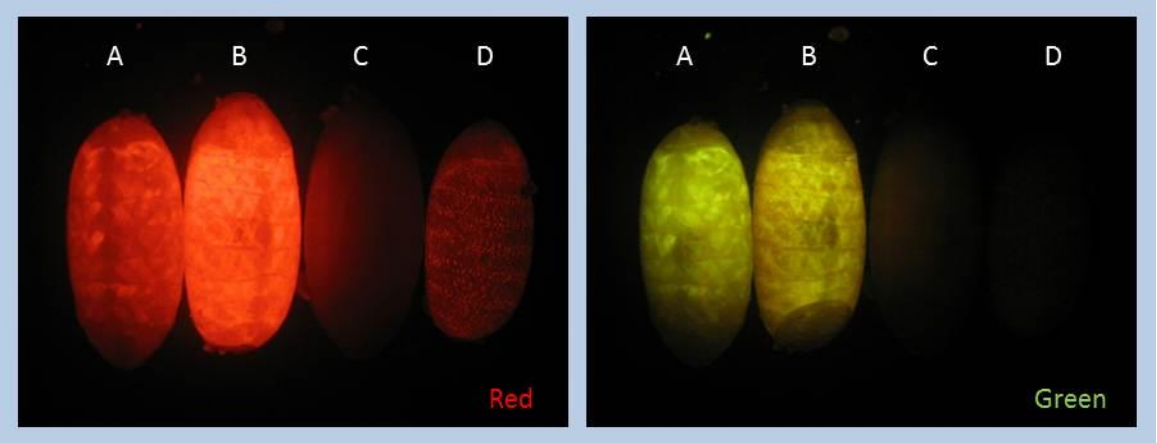


Figure 7.7 Expression of fluorescent markers in female pupae of OX4676 lines E2and L1. A – OX4676-E2-Cca, B – OX4676-L1-Cca, C – wild type, D – pupae expressing hr5IE1-driven DsRed marker. Visible DsRed and ZsGreen fluorescence in female pupae indicated successful splicing of the female-specific intron and absence of frame-shift mutations.

One possible explanation for lack of fluorescence in males is the presence of additional amino acids in the male DsRed protein. The first of three in-frame stop codons in the female-specific intron of the olive fly transformer gene starts 81 bp downstream of the exon-intron boundary, and this means the introduction of 27 aa to the C-terminus of DsRed (to avoid potential interference with splicing, no earlier stop codon was introduced into the intron sequence). This additional amino acid "tail" could potentially interfere with proper DsRed folding and impair fluorescence. This seems unlikely, given the known ability of DsRed2 to form functional fusion proteins with a wide range of polypeptides, however, were this the case it could be remedied by introduction of an earlier stop codon – for example immediately after canonical GT at the 5' end of the intron.

Another possibility is the degradation of DsRed-intron-ZsGreen mRNA in males by cellular mRNA surveillance systems. Faulty mRNA transcripts – producing secondary structures causing ribosome stalling, containing premature termination codons (PTCs), or no stop codons at all – are targeted for degradation as a way of preventing unnecessary and wasteful translation, or to avoid toxic effects of truncated proteins [53, 126]. In our case, stop codons in the female-specific *BoTra* intron could be treated by mRNA surveillance systems as premature and mRNA targeted for degradation, hence no translated protein is present and detected in the cells. However, the same stop codon is present in the female mRNA and yet females produce visible levels of fluorescent proteins. In this case removing of the intron could happen fast enough to restrict formation of degradation complexes on the mRNA so that only some mRNA is degraded while the rest is translated (this partial degradation of mRNA in females would explain weaker fluorescence of both OX4628 and OX4676 compared to the original muscleActin-driven DsRed construct). Why then did Lagos *et al.* [73] not detect lower levels of olive fly transformer mRNA in males (they did not try to detect transformer protein) – if the endogenous *BoTra* sequence stop codon in the female-specific introns is also substantially upstream from the "proper" stop codon? The mRNA degradation resulting in the lack of detectable protein would probably be noticeable even in non-quantitative RT-PCR. One explanation could be the presence of additional signals in the rest of the *BoTra* sequence – not included in our constructs – which could change the designation of the stop codon in the female intron as premature.

mRNA degradation as a reason behind lack of detectable marker fluorescence in males could be confirmed by quantitative RT-PCR. If that would be the case, introduction of an additional, exogenous polyadenylation signal in the intron

sequence may possibly help, as polyA sequences are indicated in classifying neighbouring stop codon as correct ones [38].

Alternatively, two fluorescent proteins could be expressed as two independent cassettes, with one of them disrupted by a female-specific intron and hence expressed only in females.

Chapter 8 – Creating *Ceratitis capitata* conditional male-sterile product lines

8.1. Introduction

This chapter describes attempts to create a single-construct conditional male-sterile system in *Ceratitis capitata* potentially suitable for use in a field programme, and discusses features important in such lines. At least 2 independent insertion lines were obtained and tested for sperm-sterility and fluorescent markers performance.

Based on previous experiments (described in Chapter 5) we concluded that we had sufficient information, in terms of the composition of the most important elements of the system (the driver and the effector), to attempt creating complete repressible male-sterile lines in Mediterranean fruit fly potentially useful in a field programme. From here on these lines will be referred to as 'product lines' or 'candidate product lines', by reference to the applied objectives of the CASE partner Oxitec Ltd.

There are several important aspects of the design of the construct for such a product line – some of more general nature and some more specific to this particular case. Probably the most important characteristic of such a construct is the combination of both driver and effector modules in the same transposon. This eliminates the need for performing crosses to obtain fully functional sterility

system and confines it to just one locus. Thus, in this particular case, major components of the transposon construct comprise:

- *Ceratitis capitata* β2-tubulin promoter driving tTAV expression (**driver module**)
- *Drosophila melanogaster* Protamine fused with FokI nuclease cleavage domain controlled by the tetO operator (**effector module**)
- **Transformation marker** (also useful for monitoring in the field)

Another potentially desirable feature of a product line construct is the possibility to stabilise the insertion of a recombinant DNA in the host genome. This is important not only for the performance of such a line – which to great extent may be connected to the particular genomic position of transgene in the host genome – but also for minimising environmental risks of potential horizontal transfer of integrated transposon DNA to genomes of non-target populations or species.

As mentioned before, the majority of insect transgenesis systems utilise bipartite systems comprising a non-autonomous, transgene carrying transposon lacking transposase and a separate source of a transposase [43, 102]. Transposase can be provided either as a purified protein, mRNA or as a "helper" – non-integrating plasmid containing transposase coding sequence under control of an appropriate promoter. Since transposase provided by such means does not integrate into the host genome and is very unlikely to be passed to the next generation, such non-autonomous transposon insertions can be considered stable. But the possibility of encounter with another source of suitable transposase capable of remobilisation of integrated transposon cannot be completely excluded – especially in field released insects.

While several methods have been devised to remedy the problem [48, 130], one reported by T. H. Dafa'alla *et al.* [22] seems to offer the most elegant solution as it completely removes all transposon sequences necessary for potential remobilisation. In this strategy, the transgene is flanked by two non-autonomous transposons creating a larger composite transposon. Each element – transgene and both flanking transposons - expresses different fluorescent proteins as selection markers. Because of the composite nature of such a transposon, four different integration events are possible:

- each flanking transposon integrated alone,
- the central region between flanking transposons with adjacent flanking transposons integrated – this is the intended, composite transposon
- the integration of the two flanking transposons with the plasmid backbone.

Once integration of composite transposon has been achieved – confirmed by expression of all three markers – flanking transposons can be removed by re-exposure to the transposase [*Fig. 8.1*]

Utilisation of docking sites can be used complementary to the immobilisation of a transposon. Docking sites enable site-specific integration of desired additional transgenes into a previously characterised locus. This allows modification of existing transgenic lines and eliminates the need to undertake transposon immobilisation for next transgenes. Insertion of a new transgene into the already characterised genomic locus may also help reduce the risk of influence of positional effect on new transgene expression [108].

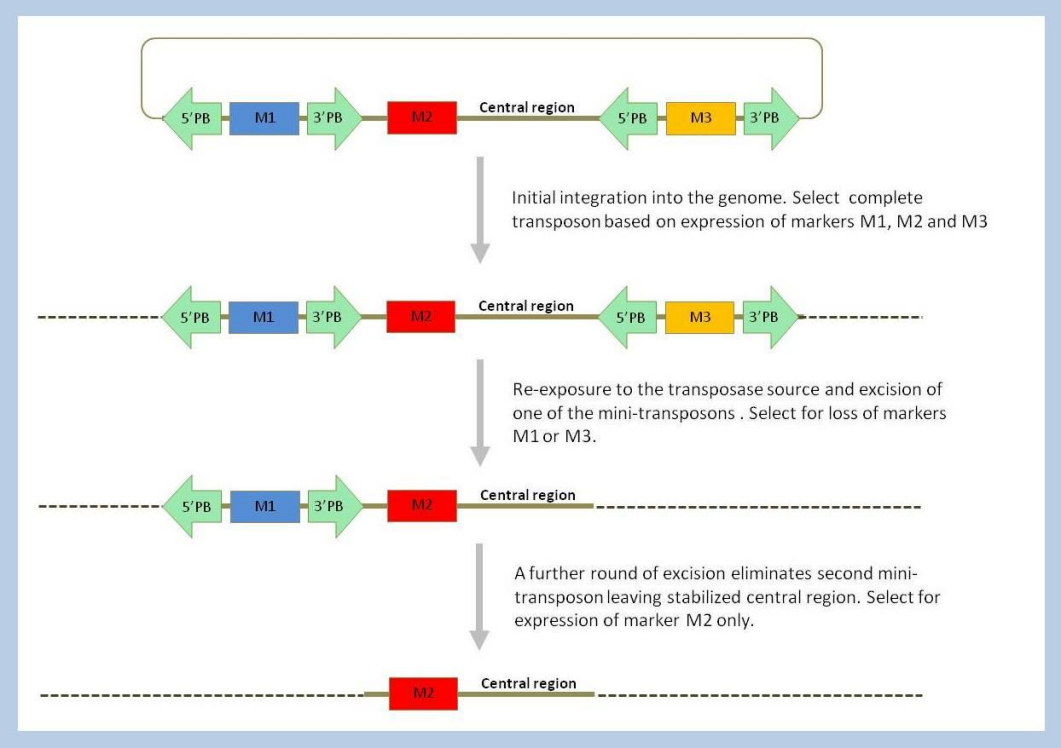


Figure 8.1 Strategy for post-integration stabilisation of piggyBac**-mediated insertions**. Introduction of shortened 5' and 3' ITRs within the original transposon creates two, smaller flanking transposons which can be excised upon exposure to the source of transposase leaving behind transposon DNA-free central region. Adapted from [22].

The choice of markers is another important feature. In the composite transposon system described above, only the marker in the central region remains associated with the stabilised transgene. As this is the marker utilised to distinguish genetically modified insects from wild type ones not only in the laboratory conditions but also (more importantly perhaps) in monitoring field released insects in SIT or RIDL programmes, it is crucial for it to be strong and long lasting. In our experience (as previously mentioned in Chapter 7), DsRed2 driven by muscle actin promoter from Mexican fruit fly possesses the characteristics demanded for such marker.

Besides whole body marking of transgenic strains, it would be desirable to be able to detect the sperm of modified released insects. It may enable larval or pupal

male selection (if clearly visible at any of these stages without the need of dissection) and identification of females mated with modified insects in the field, and could be of great help in sperm competition / precedence studies.

In our case, we decided to choose mCherry as a sperm marker. This fluorescent protein is monomeric, a feature important in our system as we prefer not to add a dimerisation domain which might increase nuclease activity, especially at low monomer concentrations. It is claimed to have increased brightness and photostability and to be suitable for fusions with other proteins [92].

Two remaining points in the design of a male-sterile construct, more specific to our particular case, are:

- Relative order and direction of the transcription of ORFs
- Presence of the multiple identical or highly similar sequences (like SV40 polyA signals or hr5-IE1 promoters) potentially allowing unwanted recombination events.

Combining transcription units on a single construct potentially allows interactions in *cis* that would not occur between separate insertions. One example is the possible formation of positive feed-back loop which increases intracellular levels of tTAV – such feed-back can occur if promoter driving expression of tTAV transactivator is placed in close proximity of the tetO operator. This might increase penetrance of desired phenotype (male sterility); however, it might also lead to unwanted, e.g. somatic, expression of tTAV (facilitated by minimal promoter between sex-specific one and tTAV cds) and subsequently to the expression of the nuclease, what can have deleterious effects on insects' fitness. Since previous experiments did not completely answer the question whether it is better to design

the sterility system with the positive feed-back loop or rather avoid it, we decided to build two versions of the candidate product construct to test.

Unfortunately, building the construct based on the original first design was plagued by unwanted recombination events, such that we were unable to obtain the intended construct. This was mainly due to the presence of multiple identical sequences of the SV40 polyA signal (some other repeats as well), which is used as the generic 3'UTR sequence in most of Oxitec's constructs. Eventually, it was necessary to replace all possible repeats, leaving only two hr5IE1 promoter sequences driving expression of fluorescent markers in the flanking transposons.

Because of the delay caused by the search for alternative 3'UTRs and promoters, only one version of the construct was completed, OX4718 – one with the positive feed-back loop. OX4718 is a composite transposon with two flanking transposons expressing AmCyan and ZsGreen as markers. The main transformation marker in the central region of the transposon is DsRed2 driven by the muscle actin promoter from the Mexican fruit fly. The driver module comprises *Ceratitis capitata* β2-tubulin promoter driving tTAV transactivator. The effector module is composed of *Drosophila melanogaster* protamine (with introns 1 and 2) fused with mCherry and a FokI nuclease cleavage domain. The central region also contains attP, loxP and FRT sites for potential future modifications of the insertion. A schematic diagram of OX4718 transposon including details of promoters, minimal promoters, 3'UTRs used and relative positions of functional elements is provided in *Figure 8.2.*

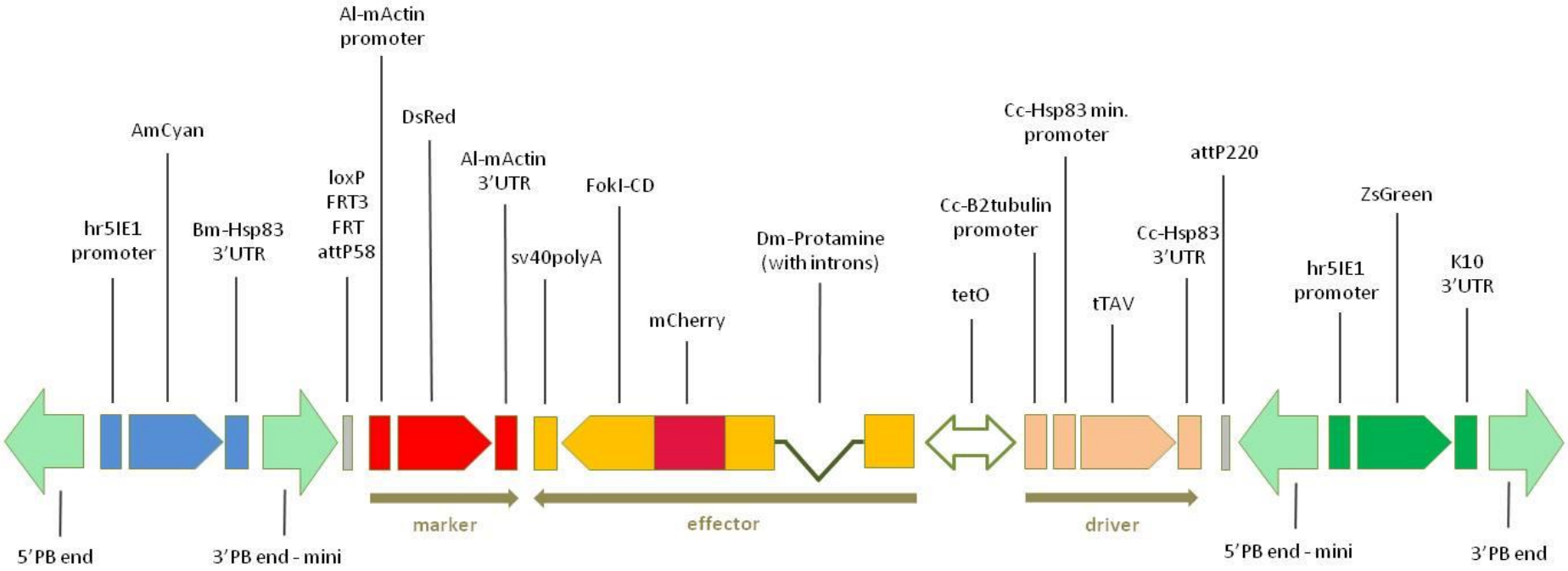


Figure 8.2 Schematic diagram of OX4718 transposon. Abbreviations used, indicating source of DNA elements: Bm – silk moth (*Bombyx mori*); Al – Mexican fruit fly (*Anastrepha ludens)*; mActin – muscle actin; Dm – fruit fly (*Drosophila melanogaster*); Cc – Mediterranean fruit fly (*Ceratitis capitata*). OX4718 transposon contains both effector (*Drosophila melanogaster* protamine fused to FokICD under the control of tetO operator) and the driver (β2-tubulin driven tTAV) on the same transposon.

8.2. Results

8.2.1. Obtaining OX4718 lines.

OX4718 plasmid was co-injected into pre-blastoderm *Ceratitis capitata* embryos together with OX3022 helper plasmid (1st day of injections) and with piggyBac transposase mRNA prepared from OX3081 plasmid (next 5 days of injections) – as described in Materials and Methods. As OX4718 carries both a driver and an effector module, the injection mix was supplemented with the tetracycline to repress expression of the nuclease in injected embryos. 8155 eggs were injected in total. Since the intention of this injection series was to produce candidate product lines, it was considered that multiple independent insertion events were required in order to be able to select the best phenotype from a larger pool – to compensate for any positional effects – hence the large number of injected eggs. Post-injection survival was as follows:

2703 larvae → 1662 pupae → 1451 adults (748 males + 703 females)

All surviving G_0 adults were crossed to wild type – in 39 crosses (in the presence of tetracycline), listed in *Table 8.1*.

Eggs were collected 2-3 times from each cross and G1 progeny were screened for DsRed and ZsGreen expression (AmCyan was not easily detectable due to low expression level and high background fluorescence).

Green only expressing pupae were found among the progeny of 10 separate G0 crosses, all but one showed a segregation pattern consistent with being single insertion events, as shown in *Table 8.2*.

OX4718-A	100 ♂ x 200 wt ♀	OX4718-N	50 ♂ x 100 wt ♀	OX4718-β	50 ♂ x 100 wt ♀
OX4718-B	50 ♀ x 20 wt ♂	OX4718-O	25 ♂ x 50 wt ♀	OX4718-γ	25 ♀ x 10 wt ♂
OX4718-C	50 ♀ x 20 wt ♂	OX4718-P	25 ♂ x 50 wt ♀	OX4718-δ	25 ♀ x 10 wt ♂
OX4718-D	50 ♀ x 20 wt ♂	OX4718-R	25 ♀ x 10 wt ♂	OX4718-ε	25 ♀ x 10 wt ♂
OX4718-E	50 ♀ x 20 wt ♂	OX4718-S	25 ♂ x 50 wt ♀	OX4718-ζ	20 ♂ x 40 wt ♀
OX4718-F	50 ♀ x 20 wt ♂	OX4718-T	25 ♂ x 50 wt ♀	OX4718-η	20 ♂ x 40 wt ♀
OX4718-G	50 ♀ x 20 wt ♂	OX4718-U	25 ♀ x 10 wt ♂	OX4718-θ	20 ♂ x 40 wt ♀
OX4718-H	50 ♂ x 100 wt ♀	OX4718-V	25 ♀ x 10 wt ♂	OX4718-λ	50 ♂ x 100 wt ♀
OX4718-I	50 ♂ x 100 wt ♀	OX4718-W	25 ♀ x 10 wt ♂	OX4718-μ	25 ♀ x 10 wt ♂
OX4718-J	25 ♀ x 10 wt ♂	OX4718-X	25 ♀ x 10 wt ♂	OX4718-π	25 ♀ x 10 wt ♂
OX4718-K	50 ♂ x 100 wt ♀	OX4718-Y	25 ♀ x 10 wt ♂	OX4718-σ	50 ♂ x 100 wt ♀
OX4718-L	25 ♀ x 10 wt ♂	OX4718-Z	25 ♀ x 10 wt ♂	OX4718-φ	20 ♂ x 20 wt ♀
OX4718-M	50 ♂ x 100 wt ♀	OX4718-α	50 ♂ x 100 wt ♀	OX4718-ψ	41 ♀ x 22 wt ♂

Table 8.1 Crosses set up with OX4718 G0 injection survivors. 1451 injection survivors were crossed to wild type flies in 39 crosses in the presence of tetracycline. Transformants expressing only ZsGreen fluorescent marker were found among the progeny of 10 of the GO crosses. Transformants expressing both DsRed and ZsGreen markers were found among the progeny of only one cross – OX4718-σ.

	G1; T	G2; T	
	transgenic	**transgenic**	**wild type**
OX4718-A	2	86	100
OX4718-C	82	103	98
OX4718-D	75	51	55
OX4718-G	59	180	196
OX4718-J	26	298	261
OX4718-K	84	208 (142 strong)	67
OX4718-N	65	157	152
OX4718-R	25	106	108
OX4718-Z	1	died at G1 as pupae	
OX4718-φ	1	died at G1 as pupae	

Table 8.2 Wild type to transgenic and male to female ratios in G_1 and G_2 in OX4718 lines expressing green marker alone. Expression of only one fluorescent marker – ZsGreen – suggests insertion of an incomplete transposon. All but one (line K) ZsGreen-only expressing lines contained single, autosomal insertions. All these lines were discontinued as they carried only a flanking transposon.

These lines were no longer propagated beyond G2 stage – samples of pupae were frozen in case they were needed for future gDNA analysis.

Pupae expressing both red and green fluorescent protein – signifying insertion of complete transposon – were found among G1 progeny of only two Go crosses: V (1 pupa) and σ (47 pupae). The single pupa from OX4718-V line did not survive to adulthood. Pupae from OX4718-σ line displayed two clearly distinct phenotypes: stronger (30 pupae) and weaker (17 pupae) and were named σ1 and σ2 respectively. These were then propagated, mostly as single male or female crosses to wild type, as listed in *Table 8.3*, to obtain unique, single insertion lines.

	G1; T
OX4718-σ1(a)	1 transgenic σ1 ♂ x 8 wt ♀
OX4718-σ1(b)	1 transgenic σ1 ♀ x 2 wt ♂
OX4718-σ1(c)	1 transgenic σ1 ♂ x 8 wt ♀
OX4718-σ1(d)	3 transgenic σ1 ♀ x 3 wt ♂
OX4718-σ2(a)	1 transgenic σ2 ♀ x 2 wt ♂
OX4718-σ2(b)	1 transgenic σ2 ♀ x 2 wt ♂

Table 8.3 OX4718 G1 transformants crosses done to obtain separate lines. Two distinct phenotypes were observed among pupae from OX4718σ line – stronger and weaker DsRed fluorescence. The two categories were named σ1 and σ2 respectively and were propagated separately, in single male (or female) crosses to isolate unique, single insertion lines.

OX4718-σ1(b) and OX4718-σ2(b) lines were discontinued at G2 stage. Analysis of G2 pupae showed that there could be more than two different insertions – the assumption originally based on two different phenotypes – in Sigma lines [*Table 8.4*].

Judging by the lower number of transgenic than wild type pupae (both "on" and "off" tetracycline) in line σ1(c) compared to line σ1(a), it may suggest that these are genuinely separate lines / insertions. This would need to be confirmed by molecular analysis of insertion sites in these lines though.

Similarly, two times more transgenic pupae than wild type ones in line σ1(d) – especially coinciding with twice the number of transgenic males compared to transgenic females – indicates an X chromosome-linked insertion, which would make it yet another, separate insertion. Line σ1(d) seemed to display two different phenotypes – stronger and weaker DsRed fluorescence – but it was difficult to be certain of the numbers at the pupal stage. The difference in fluorescence was clearly visible in 3 remaining larvae and these were used to set up separate G2 crosses.

Noticeably lower emergence rate among transgenic pupae of lines σ1(a) and σ1(c) reared without tetracycline, compared to ones reared with one, could point to some detrimental effects of the nuclease expression, most likely in somatic tissues.

G1 cross	tet	G2 pupae		G2 adults (transgenic only)	
		transgenic	wild type	♀	♂
OX4718-σ1(a)-♂	-	71	97	20	19
OX4718-σ1(a)-♂	+	109	118	53	52
OX4718-σ1(c)-♂	-	111	150	28	28
OX4718-σ1(c)-♂	+	138	172	51	62
OX4718-σ1(d)-♀	+	72	27	18	41
OX4718-σ2(a)-♀	+	86	86	31	43

Table 8.4 Wild type to transgenic and male to female ratios in G1 and G2 in OX4718 lines. Results suggest that there are more than two (σ1 and σ2) transgene insertions in OX4718 lines. Two different fluorescent phenotypes (weaker and stronger) and the pattern of transgene segregation between males and females indicates that line OX4718-σ contain two separate insertions, one of them being X-linked. OX4718-σ1(a,c) and σ2(a) lines seem to contain single, autosomal insertions.

Although, whether this is not just a rearing artefact would need to be tested more thoroughly. Lines σ1(a) and σ1(c) were reared both in the presence and absence of tetracycline as the preparation for the hatch-rate assay.

Further analysis of lines at the G3 stage (shown in *Table 8.5*) confirmed that both σ1(a) and σ1(c) lines – if indeed these are two separate lines – are single,

autosomal insertions. OX4718-σ2(a) turned out to carry a single insertion on X chromosome – as suggested by the alternating absence or presence of the transgene in males of different generations. Finally, line OX4718-σ1(d) seems to have an X-linked insertion exhibiting weaker DsRed fluorescence, and a second, autosomal insertion (with stronger DsRed expression), which appears to have a lethal effect in transgenic females when reared without tetracycline.

G2 cross	tet	G3 pupae		G3 adults			
		trans.	wild type	Transgenic		wild type	
				♀	♂	♀	♂
OX4718-σ1(a)-♀	-	110	157	19	16	n/a	n/a
OX4718-σ1(c)-♀	-	246	299	27	45	n/a	n/a
OX4718-σ1(a)-♂	+	104	114	36	39	n/a	n/a
OX4718-σ1(a)-♀	+	78	74	13	16	n/a	n/a
OX4718-σ1(c)-♂	+	116	85	44	30	n/a	n/a
OX4718-σ1(c)-♀	+	125	130	41	47	n/a	n/a
OX4718-σ1(d)-stronger-♂	-	124 s / 68 w	70	3 s / 53 w	34 s / 0 w	0	49
OX4718-σ1(d)-stronger-♂	+	132 s / 73 w	76	65 s / 65 w	62 s / 0 w	0	71
OX4718-σ1(d)-weaker-♀	-	44	33	16	14	13	10
OX4718-σ1(d)-weaker-♀	+	48	43	23	20	17	17
OX4718-σ2(a)-♂	-	100	97	84	0	0	83
OX4718-σ2(a)-♀	-	132	133	39	41	47	42
OX4718-σ2(a)-♂	+	81	84	40	0	0	39
OX4718-σ2(a)-♀	+	94	69	11	7	8	9

Table 8.5 Wild type to transgenic and male to female ratios in G3 in OX4718 lines. Results confirmed that both σ1(a) and σ1(c) lines are single, autosomal insertions. OX4718-σ2(a) carries a single insertion on X chromosome – indicated by the alternating absence or presence of the transgene in males of different generations. OX4718-σ1(d) line seems to contain two transgene insertions: an X-linked insertion exhibiting weaker DsRed fluorescence, and a second, autosomal insertion with stronger DsRed expression.

8.2.2. Testing the male sterility of OX4718 lines

8.2.2.1. OX4718-σ1(a) and σ1(c) lines

Repressible male-specific sterility caused by OX4718 transposon was first tested in lines σ1(a) and σ1(c) as these lines were first to provide sufficient numbers of progeny needed in tests. The general scheme of the hatch-rate assay was similar to

that described previously – except there was no need to obtain double-heterozygotes carrying both driver (promoter) and effector alleles.

OX4718-σ(a) and OX4718-σ1(c) lines were reared and bred either on a diet with (100 µg/ml; tet+) or without tetracycline (tet-) and were crossed to wild type. Wild type to wild type and OX4718 female crosses with wild type males in the presence or absence of tetracycline were used as controls. Fresh – not older than 24 hours – eggs from these crosses were collected starting from the day 4 after setting up cages. Three collections per cross / cage were performed. Total number of eggs was compared with the number of eggs that had failed to hatch after four days. Hatching rates were calculated as the mean percentage of laid eggs that hatched; these data showed significant sterility of OX4718-σ1 males in the absence of tetracycline [*Fig. 8.3*].

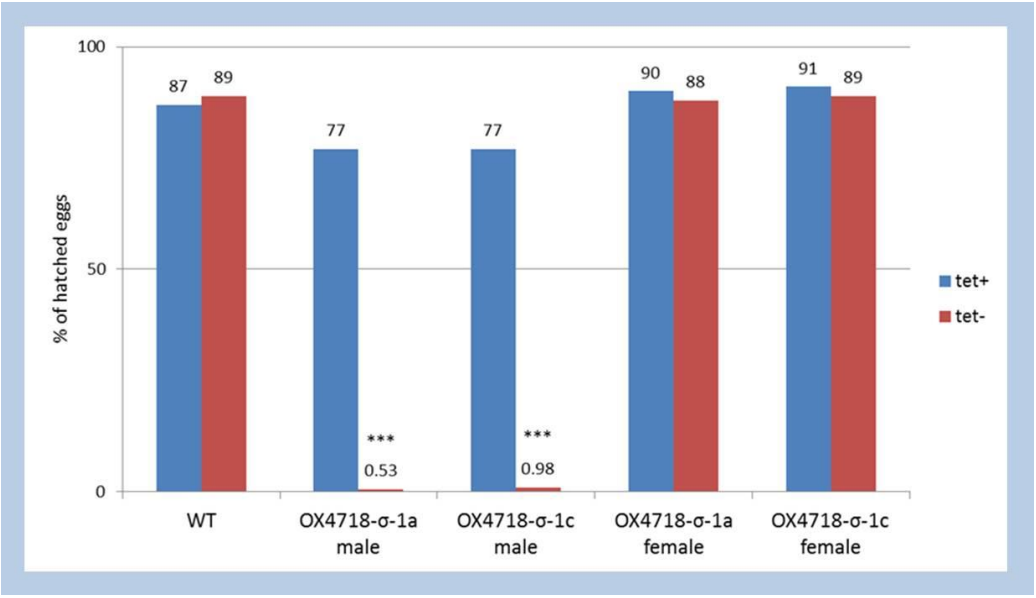


Figure 8.3 Repressible male-specific sterility in OX4718-σ1 lines. OX4718-σ1(a) and X4718-σ1(c) lines reared in the presence or the absence of tetracycline were crossed to the wild type females and the hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type to wild type and OX4718 female crosses with wild type males were used as controls. Crosses where highly significant male sterility was observed (chi-squared test, $P<0.0001$) are marked with asterisks.

Additionally, both OX4718-σ1(a) and σ1(c) males reared on a diet with tetracycline seemed to be somewhat less fertile then corresponding wild type males. It is not clear at this stage, whether this reduced fertility on tetracycline is due to incomplete repression of nuclease activity due, for example, to high expression levels, or whether it is a separate property of the line, for example and insertional effect of the transposon.

8.2.2.2. OX4718-σ2(a) and σ1(d) lines

Repressible male-specific sterility was tested next in remaining OX4718 lines – σ2(a), σ1(d)-weaker, and σ1(d)-stronger. Because σ2(a) line is an X-linked insertion, males for hatch-rate assay crosses were obtained from the female lineage. The hatch-rate assay was performed in the same way as for σ1(a) and σ1(c) lines. No female control was included in the case of the σ1(d)-stronger line. No reduction in the hatch-rate of embryos fathered by males carrying two X-linked insertions (σ2a and σ1d-weaker), reared without tetracycline, was observed [*Fig. 8.4*]. Interestingly, sibling males, when fed tetracycline, show similar decrease in fertility as the σ1(a) and σ1(c) males. This might point to an insertion effect, rather than incomplete repression as the cause, though the apparently higher fertility off tetracycline is hard to explain.

Highly significant sterility was observed, though, in the case of σ1(d)-stronger line males reared in the absence of tetracycline.

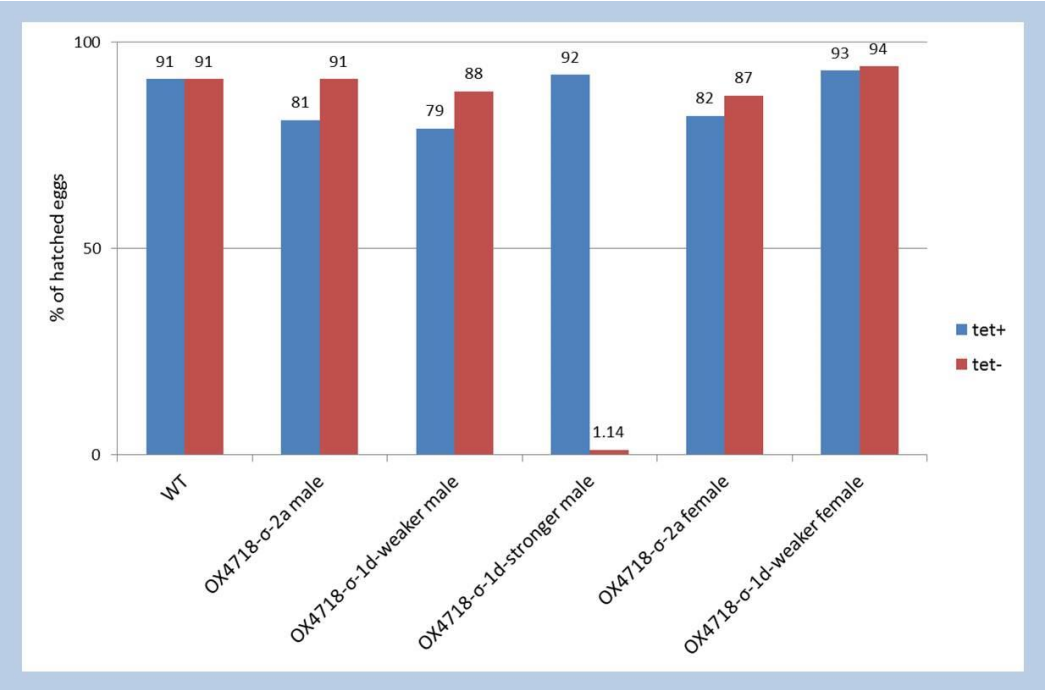


Figure 8.4 Repressible male-specific sterility in OX4718-σ1(d) and σ2(a) lines. OX4718-σ2(a) line and two OX4718-σ1(d) lines (stronger and weaker phenotype) were reared in the presence or the absence of tetracycline and crossed to the wild type females. Hatching rates of eggs obtained from these crosses were calculated (percentage of laid eggs that hatched). Wild type to wild type and OX4718 female crosses with wild type males were used as controls (except for σ1(d) stronger phenotype). Crosses where highly significant male sterility was observed (chi-squared test, P<0.0001) are marked with asterisks.

8.2.3. Performance of the transformation marker in OX4718 lines

The expression level of DsRed fluorescent marker driven by the Mexican fruit fly muscle actin promoter in OX4718-σ1 lines seems to be similar to that observed in OX4014 – the original line obtained to test this promoter. Replacement of SV40-3'UTR, used after DsRed2 in OX4014, with Mexican fruit fly muscle actin 3'UTR in OX4718 – done to minimise the risk of unwanted recombination – did not affect DsRed expression levels, at least as judged by the fluorescent microscopy. Muscle actin promoter driven DsRed proves to be a very strong transformation marker

and superior to hr5IE1 driven DsRed, commonly used in Oxitec's product lines, for example OX3864 female lethal line – as shown in *Figure 8.5*.

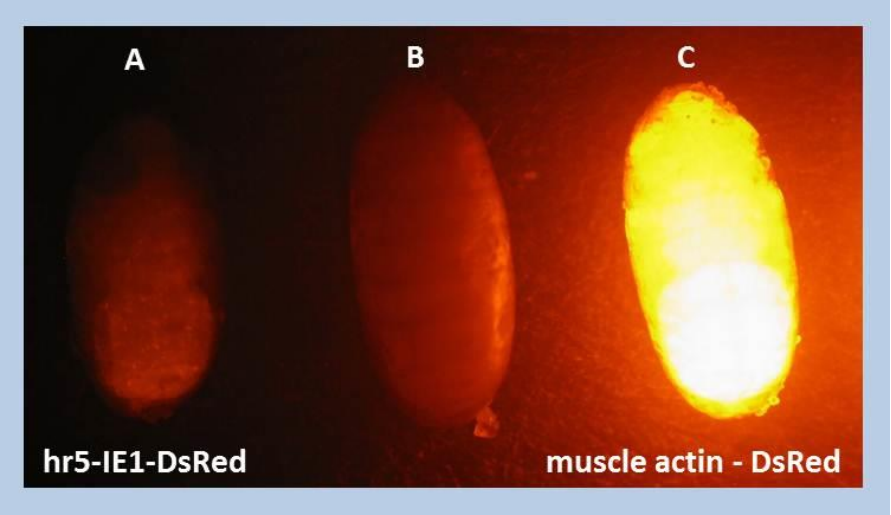


Figure 8.5 Comparison of transformation markers in OX-3864-A and OX-4718-σ1a *Ceratitis capitata* lines. A – Pupa from OX3864-Cca line; B – wild type pupa; C – pupa from OX4718-σ1a line.

8.2.4. Performance of the fluorescent sperm marker in OX4718 lines

Males and females of OX4718-σ1(a) and OX4718-σ1(c) lines used in the hatch-rate assay were tested for the performance of the mCherry fusion as the fluorescent sperm marker.

Testes from both transgenic and wild type males reared with or without tetracycline were dissected and checked for mCherry fluorescence – both as intact organ and squashed to release the sperm for more detailed examination of sperm heads. Although there was some fluorescence detectable in intact testis, as shown in *Figure 8.6*, there was none visible in sperm heads in the squashed samples [*Fig. 8.7*].

Sperm extracted from spermatheca of females mated to either transgenic or wild type males (both reared without tetracycline) also did not show any signs of fluorescence – unless stained with Hoechst DNA stain [*Fig. 8.7]*. This suggests that the red fluorescence visible in intact testis is not derived from the sperm but rather from somatic tissues and its likely source is muscle actin-driven DsRed2, rather than mCherry.

Data from hatch-rate assays indicate that the FokI cleavage domain is expressed and present in the sperm nucleus as planned. Because FokI cleavage domain is located at the C-terminus, its expression in turn indicates that the whole fusion protein is expressed, so the absence of mCherry fluorescence is surprising. Similarly, since mCherry domain is located between protamine and FokICD, frame-shift or premature termination codon mutations cannot be responsible for the lack of mCherry fluorescence. It is possible that the nuclease can induce sterility at a very low expression level, such that fluorescence from mCherry is undetectable. However, it seems more likely that the mCherry domain is simply not fluorescing as expected.

One possible reason could be that all three domains are fused without any peptide linkers. This could impair correct folding and function of mCherry. The protamine-FokICD fusion previously tested in OX4458 construct, described in Chapter 5, was also a direct one and did not contain peptide linker, but was nevertheless functional. Non-repressible constructs tested in *Aedes aegypti*, on the other hand (see Chapter 4), contained Protamine-tGFP-nuclease fusion with serine-glycine stretches (SGGGGSGGGG) in between each domain, which separated them providing more flexibility in the protein folding process.

It is possible that while both protamine and FokI nuclease are less prone to misfolding in direct fusions, mCherry may require separation with linkers for proper functioning, especially if having both N- and C-terminal fusions imposed on its structure.

Another reason for the lack of mCherry fluorescence could be the order of domains in the fusion protein. mCherry is claimed to be suitable for fusions with other proteins [92] – an important feature for the protein frequently used as the fluorescent tag helping to establish or visualise cellular localisation of other proteins. For such purposes, however, fluorescent protein is typically positioned either at the C-terminus or N-terminus of a fusion protein. In other words only one terminus of fluorescent protein is occupied by the fusion partner. In our system, mCherry domain is positioned between the protamine and FokI cleavage domain, having both termini occupied. This could interfere with mCherry function. If that would be the case one solution could be rearrangement of domains order in the fusion protein – positioning mCherry at either end of the fusion protein instead of in the middle. Especially useful could be positioning of mCherry at the C-terminal end, as the detection of fluorescence would mean that entire fusion protein is translated. This could be used in quality control of the strain. Fluorescence of mCherry positioned at the N-terminus of the fusion does not by itself confirm successful translation of the rest of the fusion protein.

It is of course possible that such rearrangements may in turn impair the function of other domains used in our fusion effector – the protamine or FokI cleavage domain. Thus, these modified effectors would have to be tested for their efficacy in inducing male sterility.

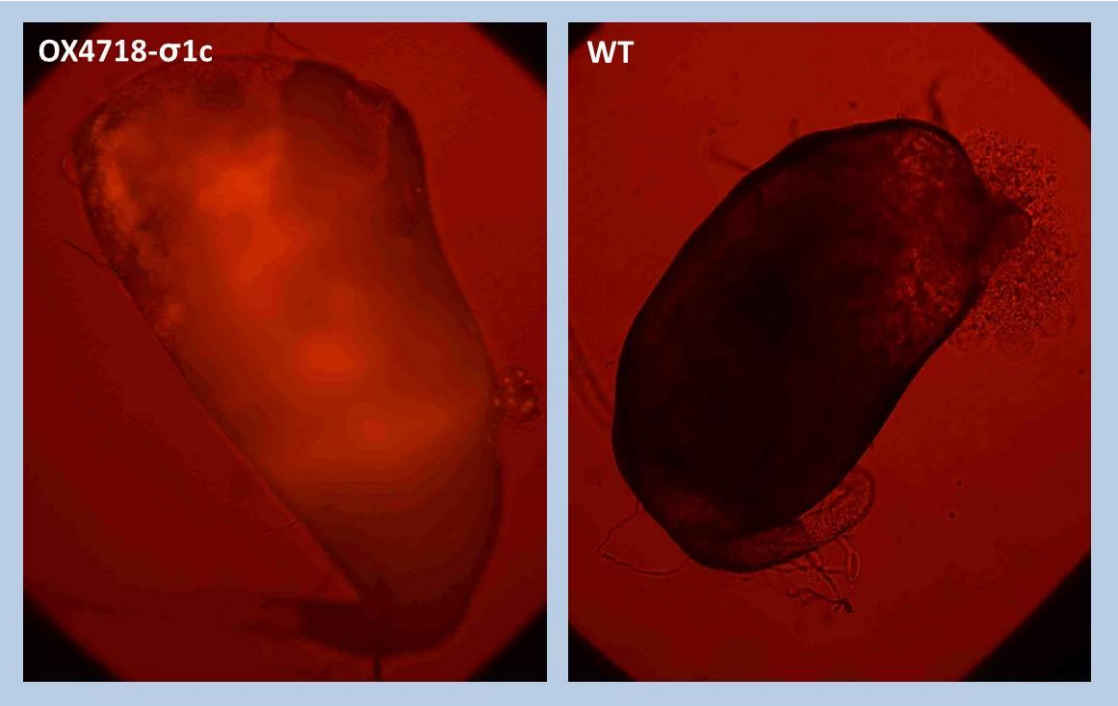


Figure 8.6 Fluorescent imaging of testis from OX4718-σ1c and WT males using red fluorescence filter. Red fluorescence is visible in the testis of transgenic males but not in the testis of wild type males. This red fluorescence does not originate in the sperm but rather in somatic tissues and its most likely source is muscle actin-driven DsRed2.

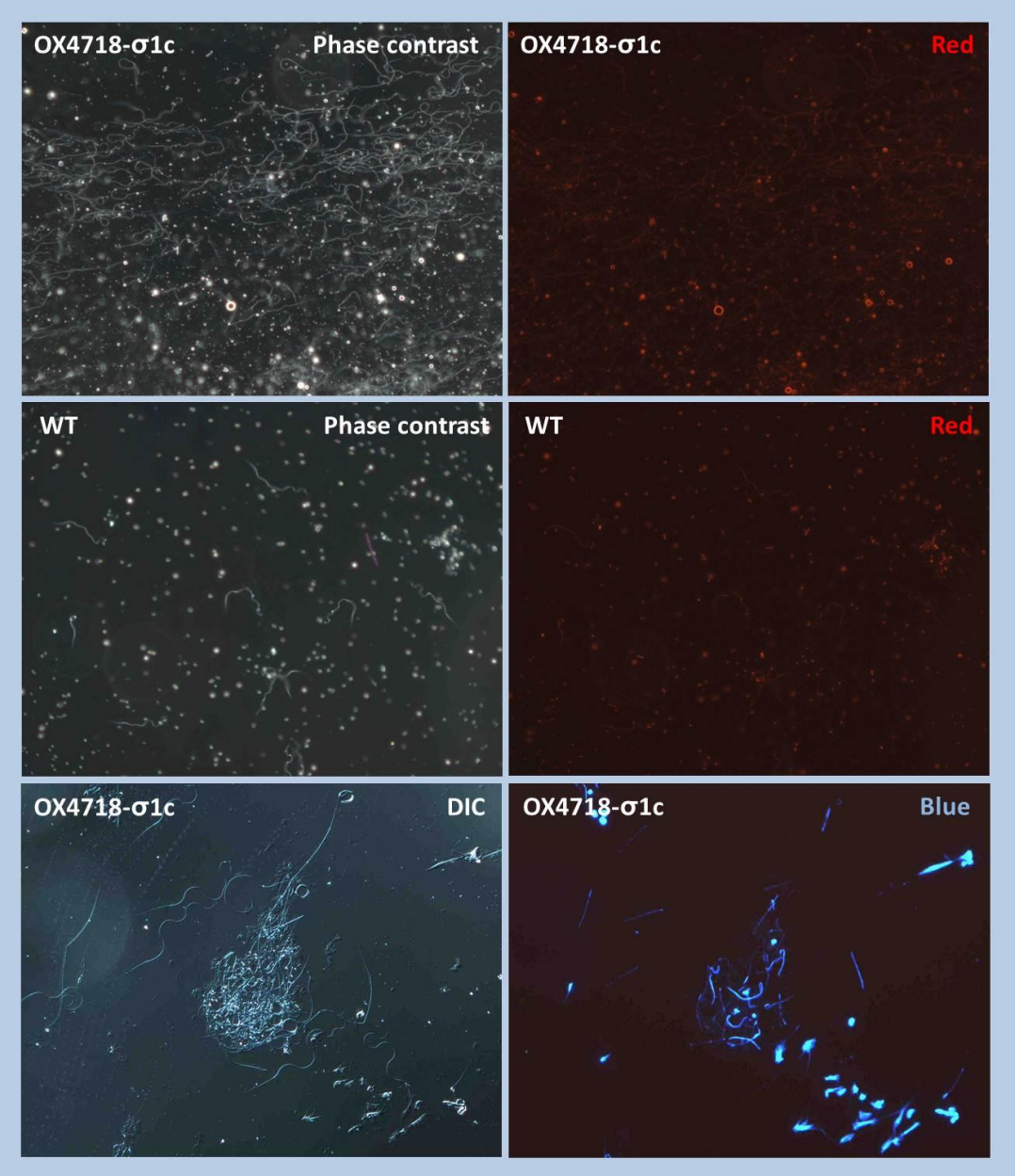


Figure 8.7 Microscopic images of sperm from OX4718-σ1c males. Four upper panels show sperm extracted from the spermatheca of females mated with off-tet reared OX4718-σ1c or WT males. Samples viewed under Phase Contrast – left panels, and under red fluorescence filter (Red) – right panels. Bottom panels show Hoechst-stained sperm extracted from the testis of off-tet reared OX4718-σ1c males viewed under Differential Interference Contrast (DIC) – left panels, and under blue fluorescence filter (Blue) – right panels.

8.3. Conclusions

The results presented in this chapter demonstrate successful development of conditional male-specific sterility in *Ceratitis capitata* in a format suitable for field use – with 2 out of 4 lines expressing the desired phenotype. Muscle actin-driven DsRed proved to be a very strong marker that could be of tremendous help in the monitoring of field released insects. What remains to be solved is the lack of mCherry fluorescence. This hopefully will be remedied by incorporation of serine-glycine spacers or by rearranging the order of domains in the protein fusion.

As previously discussed, a feature of paramount importance in SIT or RIDL programmes is the ability to release males only. Such a sexing mechanism, in the form of a female-lethal system in OX3864, was described in Chapter 7. Since an OX3864 *Ceratitis capitata* female-lethal line has already been developed and thoroughly characterised, a new, suitable male-specific sterile line was intended to be crossed with female-lethal line to produce a combined line with both sterility and sexing modules as two separate homozygous alleles. Although both constructs use DsRed as the transformation marker, different promoters used for driving it produce distinct expression patterns. In test crosses between these two lines, however, very strong DsRed expression from the muscle actin promoter completely masked expression of DsRed driven by hr5IE1 promoter, so producing such a double homozygote line would require PCR analysis instead.

A more elegant solution would be to place both modules on one transposon – this however would require *de novo* characterisation of the female-lethal phenotype.

The wide range of phenotype penetrance observed in OX4718 lines, from almost 100% sterility to none at all, indicates a strong influence of insertion site, as such,

positional effect, on transgene performance. In order to select lines with the best characteristics it may be necessary to generate more transgenic lines to choose from.

However, considering the number of injection survivors and G0 crosses and transformation rate rather few OX4718 insertions were produced. In the composite transposon, insertions of smaller, flanking "minitransposons"[*Fig.8.1*] are favoured over insertions of full transposon due to their size. This is reflected in the higher number of transgenic lines expressing only ZsGreen compared to ones expressing both markers – based on the phenotype difference, 11 "ZsGreen" lines compared to 4, possibly 5 "ZsGreen – DsRed" lines. Nevertheless, in this case, we were expecting to obtain more transgenic lines – especially ones containing only flanking transposon insertions. Such a low number of even transgenic lines expressing only green fluorescent marker suggests low overall transformation rate in this injection series, but the reason for this is not clear.

Since the majority of embryos were co-injected with the transposase mRNA, one possible explanation could be degradation or low quality of mRNA – the same preparation of helper mRNA was used in all injections. However, agarose gel electrophoresis of all the injection mixes used in the course of transposon OX4718 injections showed no mRNA degradation [*Fig. 8.8]*.

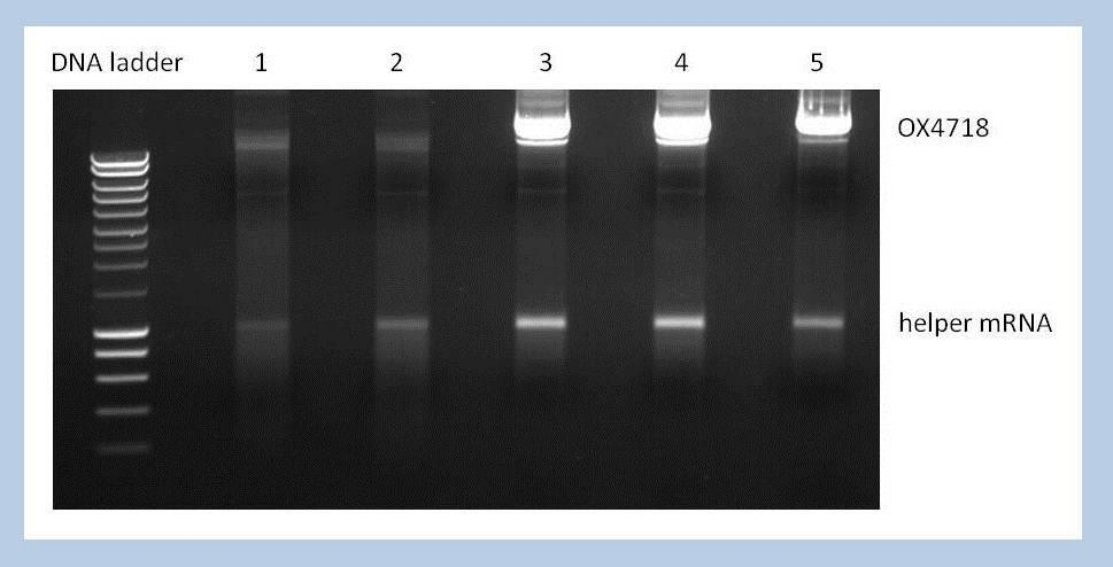


Figure 8.8 Agarose electrophoresis of OX4718 injection mixes. Aliquots of each mix were mixed with the loading buffer and frozen each day, then run together on an agarose gel.

Recombination of the OX4718 transposon also does not seem a very likely reason for the lack of composite transposon insertion. The two remaining elements in OX4718 with identical DNA sequence are hr5IE1 promoters driving expression of markers in flanking transposons [*Fig. 8.2]*. Recombination between these identical promoters would result in excision of AmCyan, both internal transposon ends (3'mini and 5'mini), and the whole central region leaving behind transposon with only hr5IE1-driven ZsGreen. Such recombination, though, would promote an even higher number of "green only" lines. If recombination happened in the plasmid before integration into the host genome, there would only be a small, very efficiently inserting transposon in the injection mix. If, on the other hand, recombination happened after integration it would alter already integrated composite transposons into small ones, expressing only the ZsGreen marker.

The size of the transposon could be another reason for low transformation rate. The correlation between the size and transformation rate has been observed in the case of the *Sleeping Beauty* transposon [134]. OX4718 is the largest transposon injected at Oxitec thus far, reaching 23 kb, nearly 3 kb larger than their second biggest, the female-lethal OX3864 construct. But despite OX3864's size, its transformation rate seemed to be on a similar level as transformation rates of smaller transposons injected in Oxitec. Additionally piggyBac is known for its large cargo capacity, recently even shown capable of delivering up to 100 kb transgenes in mouse embryonic stem cells [77]. Most of the information about size of DNA cargo integrated into the host genome via piggyBac comes from studies in mammalian cell cultures however [77, 85] – a system that has the advantage of larger numbers of integration targets (cultured cells) and elimination of cells in which transposon did not integrate. It is possible then, that in a different species context, in the intact organism, piggyBac transformation rates could be affected more by the transgene size.

If true, this may have a negative impact on future attempts to obtain transgenic male-sterile lines containing sexing mechanism (conditional female-lethal allele), which would likely be larger still. However, this needs to be verified experimentally.

Chapter 9 – Discussion

In light of increasing pesticide resistance in agricultural pests and insects of medical importance, new strategies are being developed to address these problems, for example, Sterile Insect Technique (SIT) and in particular its modern modifications, possible by the advent of genetic engineering techniques. One such proposed modification is the induction of genetic sterility due to sperm DNA damage mediated via genetic modifications, replacing irradiation traditionally used in SIT.

9.1. The goal of the project and summary of results

This project set out to design and test the conditional sterility system which relies on sperm-specific expression of DNA nucleases. The core idea of the project was based on the utilisation of a novel chimeric nuclease composed of the fusion of protamine with the FokI endonuclease cleavage domain. Both components of this nuclease effector possess properties thought to be critical for the design of the envisioned chimeric nuclease:

- Non-specific DNA binding by protamines
- Modular nature of the FokI nuclease which allows for independent functions of the recognition and the cleavage domains
- Ability of the FokI cleavage domain to non-specifically cleave DNA
- Requirement for dimerisation of the FokI cleavage domain for double-strand DNA cleavage.

These features suggested the possibility of creating a chimeric nuclease with a non-linear relationship between its concentration and activity. Such property should provide protection against leaky expression of the nuclease which, in turn, should help minimise transgenic male fitness cost. At the same, time such chimeric nuclease would exert extensive DNA damage, due to sequence non-specificity, at high concentrations in the non-repressed state. This makes our system different from others which use nucleases that cleave DNA at specific, infrequent genomic sites like I-PpoI or Zinc Finger Nucleases.

During the course of this DPhil project, we demonstrated the feasibility of inducing male-specific sterility by targeted expression of DNA nucleases in the sperm of transgenic males. Moreover, repressibility of the sterile phenotype was also achieved as a result of the implementation of the tetracycline-repressible gene expression system to conditionally drive the expression of the nuclease in the conditional fashion. Additionally, the experiments presented here demonstrated the feasibility (theoretical only at the beginning of the project) of developing a system utilising a chimeric nuclease composed of protamine fused to the FokI cleavage domain as a means of inducing male sterility.

In the system proposed at the onset of the project (Chapter 3), the activity of the envisioned protamine-FokICD chimeric nuclease was to be further controlled via a mechanism of cytoplasmic sequestration based on the GeneSwitch system [89, 99]. These attempts were unsuccessful and prompted changes in the early variants of the system and the experimental approach. One of the major problems with the testing of the GeneSwitch-type system was the high toxicity of the system inducer, mifepristone.

As a result, in next stages of the project (Chapter 4) we set out to test a simpler version of the sterility system, a non-repressible one, comprising turbo-GFP labelled *Aedes aegypti* protamine fused to either the EcoRI or the FokI cleavage domain. Cytoplasmic sequestration was omitted from these constructs to allow testing of only protamine –based chimeric nucleases. Transgenic *Aedes aegypti* males carrying this simplified sterility system showed a highly penetrant sterile phenotype. Although successful, these systems suffered from major drawback – the non-repressibility of the sterile phenotype.

Windbichler *et al.* demonstrated a similar system in which I-PpoI homing endonuclease, targeting X chromosome-linked 28S ribosomal genes, induced a strong bias toward Y chromosome–carrying spermatozoa and caused complete early embryonic lethality in *Anopheles gambiae* [131]. In their system, I-PpoI nuclease expression is under direct control of the β2-tubulin promoter. Similarly, this results in non-repressibility of the nuclease expression rendering the male line permanently sterile. Non-repressibility of the male sterile phenotype creates a problem – it is not possible to obtain homozygous, true breeding insect lines. Transgenic lines have to be maintained through the female line, outcrossed to wild type males every generation, and transgenic males have to be separated prior to release from both females and non-transgenic males.

These obstacles were overcome by utilising a conditional system, developed in the next stages of this project (Chapters 5 and 6), which allows for the temporary repression of the male sterility up until the breeding of the release generation. In the case of the system used in *Ceratitis capitata*, the key enabling step for conditional of expression was the selection of appropriate elements of the endogenous β2-tubulin promoter – removing signals responsible for the timing of

the expression of the endogenous gene, and using instead elements promoting earlier expression (5'-UTR from *Drosophila melanogaster* heat shock protein 83). As shown in the course of this project (and in the work of Dr Li Jin [61]), this shift in the timing of the promoter controlling the tTAV transactivator was crucial for activating the expression of the nuclease before the onset of meiosis and transcriptional shut down in the male germline. . The successful identification of suitable promoter elements eventually led to the creation of functional, conditional male-sterility systems in two model organisms – Yellow fever mosquito, *Aedes aegypti,* and Mediterranean fruit fly, *Ceratitis capitata.* In the latter, a partially successful attempt has been carried out in order to create conditional-sterile transgenic lines suitable for field tests. Obtained lines showed the same strong male sterile phenotype seen in previous experiments but, unfortunately, the sperm-markers included in the transposon used for the generation of these lines did not work. This would need to be addressed in the future.

We believe that the conditional sterility system developed in this project constitutes a novel modification of the SIT technology with the potential to significantly contribute to the insect control filed, especially for the control of agricultural pests. It combines characteristics of both RIDL and SIT:

- Provides an on/off switch allowing control over the expression of the nuclease effector which simplifies insect rearing. This makes it, in our opinion, advantageous in comparison to the early embryonic lethality system utilising sperm-specific expression of I-PpoI [131]
- Eliminates the use of radiation whilst simulating SIT by utilising DSBs as the lethal mechanism – which, as explained earlier, minimises the possibility of developing resistance.

- Exerts a localized effect, spatially defined by the chosen promoter – in contrast to irradiation which affects the whole insect, in particular, mitotically active cells.
- Has an in-built fail-safe property – progeny of any escapees from the rearing facility will, by default, be sterile due to unavailability of the repressor (tetracycline) in the field in quantities sufficient for the repression of the sterile phenotype.
- Although males are sterile in the absence of tetracycline, they still produce sperm capable of entering the female reproductive tract and fertilising eggs. This approach is different from the one represented by a recently developed spermless strain of *Anopheles gambiae* [119] and may be advantageous in insect species where females are known to remate which leads to competition between sperm of sterile and wild males.

Transferability of the system to new insect species would depend, among other things, on the availability of suitable promoters. These will most likely have to be isolated and tested anew in each case. Irradiation, however, would also require some fine-tuning in order to develop an SIT strain in a new species.

One major disadvantage of the conditional sterility system as developed in this project when compared to RIDL, is the lack of sexing mechanism. This, however, can be remedied by the generation of transgenic insects carrying both sterilisation and genetic sexing systems. Lastly, in RIDL programmes based on sex-specific, female lethality, sons of the released males survive and pass the lethal allele to the next generation killing half of their daughters. This further adds to the effectiveness of the programme. However, a similar effect could probably be achieved with the conditional sterility system by selecting strains with a partially

penetrant phenotype. This would imitate the so called inherited sterility in which sub-sterilising doses of ionizing radiation delivered to the F0 generation cause an inheritance of radiation-induced deleterious effects by the next generation (F1). This results in reduced number of hatched F1 eggs and highly sterile F1 offspring [88, 111].

9.2. DNA double-strand breaks as possible explanation of male sterile phenotype

It is not yet certain, however, if the observed phenotype is indeed caused by double-stranded breaks in the sperm DNA induced by protamine-FokICD chimeric nuclease. How likely is it then that the sperm sterility is indeed due to the proposed mechanism? We do know that when expressed, turboGFP-labelled protamine was localised to sperm nuclei – as indicated by fluorescence in sperm heads of sperm dissected from *Aedes aegypti* males carrying the OX3879 construct. These males did not exhibit the sterile phenotype. Adding either the EcoRI or FokI cleavage domain to protamine-tGFP fusions resulted in highly penetrant sterility in transgenic males expressing these nuclease fusions. Localisation of the expressed nuclease fusion proteins in the sperm of these males remained the same as confirmed by detection of tGFP fluorescence in sperm heads. The same phenotype observed in transgenic males expressing either EcoRI or FokI cleavage domain fused to tGFP-labelled protamine suggests similar molecular mechanisms. Nucleus-targeted expression of EcoRI has been shown to cause DNA cleavage and cell death in *Saccharomyces cerevisiae* [7]. Similar results – DNA cleavage – were observed in

Saccharomyces cerevisiae mitochondria due to targeted overexpression of EcoRI endonuclease in the organelle [118]. It is therefore probable that the male sterility observed in insects expressing protamine-tGFP-FokICD fusion proteins is caused by DSBs introduced by the fusion nuclease. From experiments with transgenic insects carrying OX4458 and OX4627 constructs in Mediterranean fruit flies and *Aedes aegypti* mosquitoes respectively, it is also clear that no mediation of dimerisation of protamine-FokI through additional domains is required. Both these constructs contain the coding sequence for the protamine-FokICD fusion protein, without fluorescent protein marker, as a nuclease effector. Double heterozygous insects carrying OX4458 / OX4627 allele and driver (promoter-tTAV) alleles showed similar, highly penetrant male sterile phenotype in the absence of tetracycline.

There are also some similarities between the structures of the FokI-DNA and protamine-DNA complexes suggesting that protamine-FokICD fusion could cause DNA cleavage. Both FokI and protamines bind to the major groove of DNA and both have similar DNA footprint [5, 11, and 128]. Hence, both the native FokI and the protamine-FokICD fusions can probably position the FokI cleavage domain in a similar way in relation to the DNA helix. In addition, as mentioned in Chapter 1, DNA-protamine complexes are stabilized by protamine-protamine interactions. DNA-bound protamines form both inter- and intra-molecular disulphide bridges which help with the formation and maintenance of a highly condensed chromatin structure [4]. This close contact between the different protamine molecules suggests that the protamine-FokI fusion proteins should be able to bring two FokI cleavage domains close enough to facilitate possible dimer formation.

There is also another characteristic of FokI that makes induction of DSBs by protamine-FokICD probable. In the native FokI endonuclease, the cleavage domain is bound to the DNA recognition domain. This sequestration, together with the dimerization of cleavage domains, serves as a safe-guard against non-specific DNA cleavage / nicking during the scanning for correct the sequence [15, 123, 127 and 128]. However, Li *et al.* using proteolytic assays have shown that the purified FokI cleavage domain, without the recognition domain, was capable of non-specific DNA cleavage in *in vitro* assays [76]. Similarly, Bitinaite *et al.* showed that the cleavage domain alone was also able to enhance the cleavage rate when mixed with rate-limiting concentrations of wild-type FokI, but they could only restore wild-type cleavage levels at a very high molar excess [15].

This data suggests that the FokI cleavage domain, if not bound by the recognition domain, is in an active state requiring only dimerization with another cleavage domain for the successful digest of double-stranded DNA. This data, together with the properties of protamines discussed above, indicate that it is quite possible for protamines to mediate FokI cleavage domain dimerization leading to non-specific DNA cleavage.

9.3. Detection of DNA double-strand breaks

To unambiguously establish the presence of double-strand breaks in the sperm DNA of transgenic sterile males and to demonstrate the suspected molecular mechanism of the genetic sterility system, additional tests will be required.

One method of detection of double-strand breaks, developed specifically for SIT programmes, relies on the comparison of sperm head size between sterilized and

wild type (or wild) males. For example, it has been shown that sperm heads of irradiated Mediterranean fruit fly males are slightly shorter (20.5 – 29.0 μm) than sperm heads of wild, non-irradiated males (27.5 – 33.0 μm) [82]. The purpose of this method is to measure more directly the mating quality of released insects by identifying sperm extracted from spermathecae of trapped wild, mated females using darkfield microscopy [82]. This helps establish whether each trapped wild female has mated with a sterile male, a wild male or both.

The method is based on the finding that the size and shape of the sperm head is related to the amount of genetic material present in the sperm nucleus. Chromosomal abnormalities, caused by DNA double-strand breaks, may lead to mispairing during meiosis and loss of chromosomes. This results in smaller sperm heads [41, 51, and 104]. It implies that, for this method of DSBs detection to work, it is required that DSBs are introduced before the onset of meiosis. Post-meiotic induction of DSBs in transgenic mosquitoes carrying the non-repressible sterility system described in Chapter 4 would, therefore, not result in changes in the sperm head size. It is also not certain if the activity of the protamine-FokICD chimeric nuclease from the conditional systems described in Chapters 5, 6 and 8 is present sufficiently early in order to result in the loss of chromosomes during meiosis and the reduction of sperm head size.

Another method of detection of DSBs utilizes the cellular response to damage of genetic material and was used by Windbichler *et al.* to confirm the mechanism of action of their non-repressible X-chromosome targeting system [131]. Among the earliest cellular responses to double-strand breaks is the phosphorylation of a variant of histone H2A, histone H2AX. This modification is used as a signal for the recruitment of the DNA-repair machinery. Phosphorylated histone H2AX (referred

to as γ-H2AX) can then be detected with phosphorylation-specific monoclonal antibodies, indirectly indicating the presence of DSBs [75, 131, and 132].

It is not entirely clear whether DNA cleavage induced in our sterility system will coincide with the presence of histone H2AX, and therefore whether γ-H2AX detection can be used as a test for the presence of DSBs. One way to get around this potential problem would be to detect γ-H2AX in embryos fertilised by transgenic males rather than in the sperm of these males. DSBs, carried over to the zygote, would likely invoke the DNA-repair response by the zygote's cellular machinery. In fact, this approach was used by Windbichler *et al.* in their experiments [131].

However, as DNA fragmentation is more and more recognized as an important indicator of sperm quality and one of predictors of IVF treatment outcomes, several other assays are now available to test DNA fragmentation. Some of these were developed with medical / clinical use in mind, but, most likely, they can be adapted to the particular needs of this project. These alternative methods for detection of double-strand breaks include:

- TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labelling) – allows direct detection of single and double-stranded DNA breaks by labelling terminal nucleotides at the site of cleavage [39].
- Comet assay – microgel electrophoresis based assay in which DNA fragmentation is detected as increased DNA mobility of gel-encapsulated samples. The more fragmented the DNA sample is, the higher its mobility in the gel, producing a characteristic comet shape when visualised using fluorescence microscopy [20]
- SCD test (sperm chromatin dispersion test) – bright field microscopy, agarose microgel based detection of DSBs. Following acid denaturation,

fragmented DNA is distinguished from non-fragmented by the lack of the chromatin decondensation halo [31].

9.4. Future directions of the project

The identification of the elements necessary for the proper function of a conditional male-sterile system and the demonstration that they can be successfully put together to achieve the desired phenotype is only the first step in the development of transgenic lines intended for deployment in the field.

In the case of the Mediterranean fruit fly system presented here, in the most immediate future, the issue of a non-functional sperm marker would need to be addressed. The presence of a functional fluorescent sperm marker can offer an improvement of the monitoring of the SIT (or RIDL) programme and allow for easier assessment of mating success of the released males [110]. Information on which male has mated with the wild female – released transgenic male, wild male, or both – is important in assessing the efficiency of SIT programs. Current methods for analysing the mating status of trapped wild females rely on checking the progeny of mated females [67], comparing the length of the sperm heads of spermatozoa obtained from mated females since sterilised sperm heads are shorter then wild type [82], or using the PCR technique [103]. Since all these methods are rather cumbersome and laborious, the use of a fluorescent sperm marker can thus be advantageous.

The crucial aspect of the SIT program is the ability to release a population of pure males into the environment. This minimises crop damage (no extra oviposition wounds) in case of agricultural pests and prevents the increase of health risks in case of disease vectors like *Aedes aegypti*. Hence, incorporation of the previously mentioned female-lethal RIDL system into the male-sterile lines – enabling separation of males from females – would greatly improve such strain. As both OX4718 and (male-sterile) OX3864 (female-lethal) *Ceratitis capitata* lines use DsRed as the transformation marker and crossing these two lines to generate a double homozygous line may prove to be difficult, a more elegant and efficient solution would be to place both transgenes into a single transposon.

The next steps in the development of transgenic male-sterile lines, would involve:

- characterisation of the insertion sites, for example by inverse PCR [122] or by oligo-cassette ligation mediated PCR [101],
- stabilisation of the transgenes by removal of the flanking transposon ends ,
- obtaining true breeding, homozygous lines.

As the process of removing the transposon ends or creating true breeding lines is not always straightforward, and its success may depend on positional effects of a particular insertion site (for example insertion into highly repetitive sequence which makes obtaining a homozygous line quite difficult), a panel of several transgenic lines with confirmed sterile phenotypes would be desirable.

The ultimate test for the effectiveness of the male-sterile lines is their field performance. As the success of the SIT program depends on sterile males and wild females mating, the ability of sterile males to compete against wild males, in other

words, the fitness or quality of insects is crucial. In the SIT programs, there is a direct relationship between the complexity of the mating system of particular targeted insect species – and associated level of competition for mates – and the importance of producing competitive, fit males. Very simple mating systems, with little competition for mates, will require less stringent quality control of the produced sterile males. Different features of mating systems influencing levels of competition for mates, and hence making insects with such mating system easier or more difficult to control with SIT, are listed in *Table 9.1*.

Characteristic of mating system	**Favourable**	**Unfavourable**
Male courtship ritual	Simple	Complex
Female choice of mates	Passive (accepts first male)	Active (chooses among males)
Sex pheromone	Female-produced, simple	Male-produced, complex
Characteristics of adult male	Long-lived, active disperser	Short-lived, sedentary
Male-male competition	Indirect (scramble for mates)	Contest for mates or resources
Mating in time and space	Distributed throughout habitat, asynchronous	Highly aggregated, e.g. lek system in medfly

Table 9.1 Characteristics of insect mating systems that are favourable or unfavourable for the development and operation of programmes releasing sterile insects. (*Adapted from "Sterile Insect Technique – Principles and Practice in Area-Wide Integrated Pest Management". V.A. Dyck, J. Hendrichs and A.S. Robinson, Springer, 2005*)

It was stated in the introduction that male-sterile systems based on inducing double strand breaks in chromosomes minimise the risk of resistance against such sterility. It would be indeed difficult to envision some form of molecular, genetic

resistance mechanism against such system. However, other forms of resistance, for example behavioural, remain possible. In insects with mating systems based around active female choice of mates, like *Ceratitis capitata* [72], females could, upon sensing the lower fitness of transgenic males, preferentially mate with wild males. Such female choice, leading to assortative mating, would significantly decrease the effectiveness of the SIT programme.

The fitness of insects can be affected by a variety of factors. Some are more general, affecting not only transgenic but all insects which are artificially bread, for example, rearing conditions, genetic makeup of the particular strain etc. Other factors will be more specific for genetically modified insects, for example, the mutational effects of transgene insertion into the genome [58, 79]. The fitness aspect specific for our particular case is the quality of produced sperm, or more generally, the quality of the ejaculate. Mediterranean fruit fly transgenic males producing sperm / ejaculate of lower quality, when compared to wild males, may not be capable of inducing refractoriness in females after mating. This could lead to females remating with wild males resulting in the production of viable progeny, and hence, compromising effectiveness of the control programme. The number of eggs produced by females mated with transgenic males in experiments conducted in this project, or during microscopic assessment of sperm from transgenic males, do not indicate such problems. However, transgenic males obtained in the course of this project never had to compete for mates against wild type ones. A more thorough assessment of sterile-male lines, testing their mating competitiveness and assaying sperm quality in sperm competition assays, is needed.

Similar steps would also need to be undertaken in future work on the *Aedes aegypti* conditional sterility system. So far, the issue of sexing-mechanism, best suited for implementation into the sterility system has not been fully resolved and would need to be tackled next. Currently, the flightless, late acting *Aedes aegypti* female is available [36]. However, at this stage it is uncertain if it would be better than an early acting female-lethal system. Sperm competition, or remating assays will be useful as well, albeit less crucial than in *Ceratitis capitata*, in light of the results by Thailayil *et al.* [119] which reports the induction of female mating refractoriness by spermless males in *Anopheles gambiae* [119].

Additionally, the system presented here could be useful for inducing male-sterility in other organisms, for example, Lepidoptera, which are poorly amenable to the classical SIT method. Because expression of the nuclease in the conditional sterility system developed during this project is confined to the male germline, even a very high number of double-strand breaks in DNA should not affect somatic tissues. In other words, much higher levels of DNA damage can be achieved without affecting insect fitness than possible when using radiation sterilisation. It is believed that the reason behind Lepidoptera radiation resistance lies in the diffused centromeres on their chromosomes (holokinetic chromosomes) [71]. In that case, one or very few DSBs per holokinetic chromosome, introduced by a non-debilitating dose of radiation may not be enough to lead to loss of separated chromosome fragment(s) during subsequent early embryonic cell divisions. Nuclease based conditional sterility systems may be used to overcome such obstacles.

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