Development of genetic control methods in two lepidopteran species

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Abstract

The ability to genetically engineer insects has opened the door to the development of new pest control techniques, notably the improvement of the sterile insect technique (SIT), in which radiation-sterilised insects are mass-released to suppress the wild population. Genetics-based methods may provide an alternative to irradiation, which can decreases the competitiveness of sterilised insects, by engineering repressible dominant lethality, a technique known as Release of Insects carrying a Dominant Lethal (RIDL®). Gene transfer and site-specific recombination technologies have here been developed in two lepidopteran pests, as the first step in the development of genetic control methods. The diamondback moth, *Plutella xylostella*, is a major pest of cruciferous crops and is difficult to control, mainly due to widespread resistance to insecticides, so the development of new control measures is needed. The first germline transformation of the diamondback moth has been achieved, using the *piggyBac* transposable element. The functionality and utility of the ΦC31 integrase, Cre and Flp recombinases in the manipulation of the diamondback moth genome has been demonstrated, and the use of recombinase-mediated cassette exchange using *lox* and *FRT* heterospecific sites has been investigated. The pink bollworm, *Pectinophora gossypiella*, is a significant pest of cotton. In south-western USA this moth is a target of SIT; the development of a pink bollworm female-sterile strain would remove the need for sterilisation by irradiation. Towards this end, *Bombyx mori* ovary-specific regulatory sequences have been tested in pink bollworm, and their potential use in the development of conditional female-sterility investigated.

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Author's declaration

The research reported within this thesis is my own work except where otherwise stated, and has not been submitted for any other qualification or degree.

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Introduction

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1.1.1. The biology and life history of the diamondback moth

The diamondback moth, *Plutella. xylostella* (L.), is a widely distributed and damaging pest of wild and cultivated Cruciferae plants. Host plants such as cabbage, broccoli, cauliflower, mustard and rapeseed are the favourite hosts (Campos, 2006).

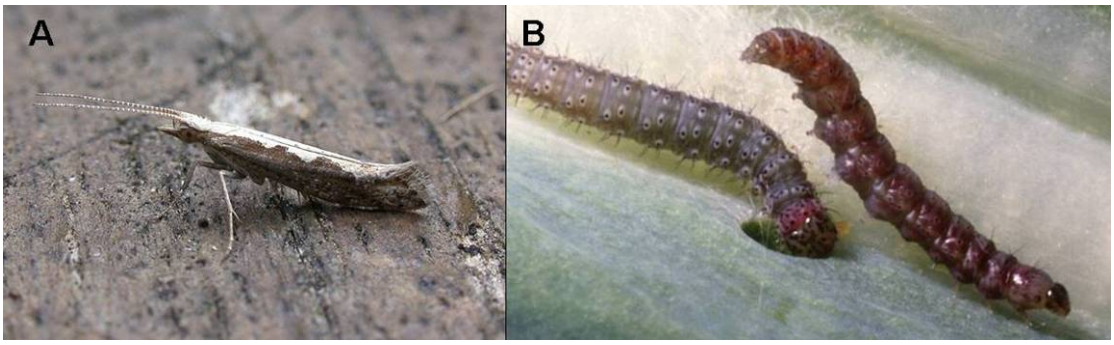

Figure 1.1. The diamondback moth. (A) Adult, (B) late larvae.

The life cycle of the diamondback moth includes four stages of development: egg, larva, pupa and adult. Oviposition usually begins on the day of emergence and one female can deposit up to 356 eggs (Harcout, 1957). The eggs are laid singly or in small groups on the leaves of the host plant. Newly hatched larvae crawl to the underside of the leaves where they bore into the epidermis and feed internally on the plant tissues. At the end of the first instar, the larvae emerge from their mines and feed externally in all subsequent 3 instars. Pupation takes place inside a loose silk cocoon on the underside of the leaf or in the ground. Adults are small and slender moths that feed on flowers of the Brassicae, water drops or dew (Talekar & Shelton, 1993) and are nocturnal in behaviour (Figure 1.1). In ideal condition, the moth's life cycle averages 25-30 days with many overlapping generations throughout the year in

warmer climates. The insect does not survive rigorous winters, although it can develop from about 10-35ºC (Campos & Schoereder, 2006) with no clear differences in female egg production (Shirai, 2000).

1.1.2. Distribution of the diamondback moth

As cultivated brassicas are considered of European origin, it was accepted that the diamondback moth is of European origin, from the Mediterranean area (Talekar & Shelton, 1993) and spread with the cultivated brassicas around the world. Based on studies on the diversity of the diamondback moth parasitoids and diversity of indigenous host plants, however, it has been suggested that the diamondback moth may have originated in southern Africa (Kfir, 1998). It now occurs wherever crucifers are grown, and this insect is believed to be the most widely distributed of all Lepidoptera (Talekar & Shelton 1993).

1.1.3. Damage caused by diamondback moth

Diamondback moth larvae are serious defoliators. Larvae feed on the lower leaf surface and all leaf tissues are consumed except the veins. This is particularly damaging to seedlings. Larvae can also feed on other parts of the plant such as florets of broccoli and cauliflower and on cabbage heads, disrupting head formation.

1.1.4. Control of the diamondback moth

The diamondback moth is difficult to control, mainly due to resistance to insecticides. The absence of effective natural enemies, especially parasitoids, the ability of

diamondback moths to migrate long distances (1000 km/day) and the destruction of natural enemies with the use of broad-spectrum insecticides have also been highlighted as factors that make diamondback moth control difficult.

1.1.4.1. Chemical control

Insecticide applications to control the diamondback moth cost an average of $123 per hectare (Reddy & Guerrero, 2000), with yield losses reaching 31% (Macharia et al., 2005). The diamondback moth was the first insect to become resistant to DDT – in Indonesia in 1953, three years after introduction of the insecticide (Arkersmit, 1953). In the 1980s, pyrethroids began to fail and the moth soon acquired near-immediate resistance to all categories of insecticides (Shelton et al., 1993). In the early 1980s, insect growth regulators (IGRs) were introduced and provided an efficient alternative to synthetic insecticides. However, the diamondback moth also developed resistance to some IGRs such as chlorfluazuron, duflubenzuron, hexaflumron (Talekar & Shelton, 1993). Besides development of resistance, pesticides are associated with other problems. Due to the amount of wax in the surface of the leaves, in particular those of cabbage, it is difficult for pesticides to remain on the leaf surface. Furthermore, early instar larvae live within the plant tissue, sheltered from the pesticides.

A more recent alternative to synthetic insecticides is the development of botanical insecticides. Some plant allelochemicals are feeding deterrents; make the plant unpalatable to the larvae that then die of starvation. Neem (*Azadirachta indica*) has been used against the diamondback moth and has been shown to be an oviposition deterrent and to have antifeedant and growth-regulating activity (Facknath, 1997).

Neem has also been found to be harmless to larval parasitoids of diamondback moth (Facknath, 1997; Krishnamoorthy, 2004). To avoid resistance, chemicals from different pesticide families have been used in rotation.

1.1.4.2. Biological control

All stages of the diamondback moth are attacked by numerous parasitoids and predators (Talekar & Shelton, 1993). More than 90 parasites of the diamondback moth have been recorded in various parts of the world (Noyes, 1994; Talekar & Shelton, 1993). However, only very few were found to be effective (Noyes 1994, Krishnamoorthy, 2004) in food crop plantations.

Egg parasitoids belonging to the genera *Trichogramma* and *Trichogrammatoidea* have been reported to attack the eggs of diamondback moth in many parts of the world. In practice, however, they contribute little to control and have the disadvantage of intolerance to low temperatures (Krishnamoorthy, 2004) and require frequent mass releases. Larval parasitoids belong mainly to the genera *Diadegma* and *Cotesia,* and seem to provide good control (Talekar & Shelton, 1993). *Cotesia plutellae* Kurdj is the most dominant larval parasitoid. In India, it tends to be ineffective due to a lack of synchronisation between its occurrence and diamondback moth occurrence (Krishnamoorthy 2004). Introductions of *C. plutellae* in Antilles (Bennett & Yaseen, 1972), Papua New Guinea (Saucke et al., 2000) and Taiwan (Talekar & Shelton 1993) did not provide adequate control. However, it has been successfully introduced in Australia (Hamilton, 1979) and Cape Verde Islands (Talekar & Shelton, 1993). In addition, some *Diadromus* spp. that attacks the pupae of diamondback moth were successfully introduced in New Zealand (Talekar & Shelton, 1993).

More than 10 species of predators are known (Bauer et al., 1995), although predators are not as well studied as parasitoids. Examples of these are *Motacilla flava* (yellow wagtails) and a few ants such as *Tapinoma melanocephalum*, *Pheidole spp*., *Componotus sericeus* (Krishnamoorthy 2004) and *Chrysoperla carnea* (Reddy & Guerrero, 2000).

Fungal pathogens such as *Paecilomyces farinosus*, *Zoophthora radicans*, *Beauveria bassiana* and *Vericillium lecanii* (Sarfraz et al., 2005; Krishnamoorthy, 2004) are known to infect the larvae and pupae of diamondback moth. For instance, *P. farinosus*, can kill the larvae just 48-72 h after infection (Krishnamoorthy, 2004). Bacteria such as *Bt* and viruses such as the *P. xylostella* granulosis virus also have the potential to aid diamondback moth control.

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae have considerable potential to control insect pests. However, their application is difficult as they are susceptible to UV radiation, extreme temperatures and rapid desiccation. Among these nematodes, *Steinernema carpocapsae* is one of the most promising against diamondback moth due to its highest resistance to desiccation compared to some other nematodes (Bauer et al., 1995).

1.1.4.3. Host resistance

First-instar larvae avoid leaves with high wax content, which can result in larval starvation and desiccation (Eigendrobe, 1990). Resistant glossy varieties of cabbage, with high wax content and lacking the normal waxy bloom, have been developed to control the diamondback moth. Glossy type cabbages provided over 95% of control of the diamondback moth (Dickson et al., 1990).

The diamondback moth was the first insect to develop resistance to *B. thuringiensis* var. *kurstaki* (McGaughey, 1994) in the field. *Bt* plants containing the *aizawai* strain instead of the *kurstaki* serotype have been developed. As the *aizawai* serotype has more toxins then the *kurstaki* serotype and there is no evidence of cross-resistance between some serotypes of *Bt* (Talekar & Shelton, 1993), these strains are expected to provide good diamondback moth control.

1.1.4.4. Cultural control

Rainfall has been identified as an important factor in mortality for the diamondback moth (Talekar & Shelton, 1993; Harcourt, 1963). The eggs and larvae are washed off from the plant or drown (Facknath, 1997). Overhead irrigation (Sprinkler irrigation) has been shown to reduce the number of diamondback moth in cabbage (Talekar & Shelton, 1993) and watercress (Tabashnik et al., 1986). Applications of overhead irrigation at dusk also affect the adults by reducing mating-related flight activity. The downside of these operations is their high cost and encouraging the development of diseases, such as black rot and mildew.

Intercropping where diamondback moth host plants are alternated grown with plants, such as sage, thyme and white clover, that are not susceptible to the moth (Talekar & Shelton, 1993), makes it more difficult for the adult moths to find new host plants that are camouflaged between other non-susceptible crops. Non-susceptible crops act as physical barriers to the movement of the moths, disrupt olfactory and visual cues between insects and between the insect and the host plant, and favour the establishment of natural enemies (Talekar & Shelton, 1993). The success of

intercropping cabbage with tomato (Buranday & Raros, 1973; Talekar & Shelton, 1993), and cabbage with garlic (Talekar et al., 1986), has been reported.

Trap cropping consists of planting plots of less important plants that are more attractive to the diamondback moth than the main crop being grown, in order to draw the moths away from the main crop. Mustard and rapeseed are the most frequently used and successful trap crops (Facknath, 1997; Talekar & Shelton, 1993). Trap crops should be monitored closely as unattended trap crops can generate large populations of diamondback moth. Crop rotation can be used to disrupt continuous generations. Planting seedbeds distant from production fields and removal of unharvested plant parts where the diamondback moth can survive are other cultural practices that can help to reduce the population of diamondback moth.

1.1.4.5. Mating disruption

In 1974, Chow et al. reported the sex pheromone released by the female diamondback moth. The major components of the diamondback moth pheromone are (*Z*)-11-hexadecenal (*Z*11-16:Ald) and (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) (Reddy & Guerrero, 2000; Talekar & Shelton, 1993; Chisholm et al.; 1979). The synthetic pheromone, placed inside a trap, has been utilised to monitor pest populations in control programmes and has been used successfully in mating disruption in Japan (Talekar & Shelton, 1993).

1.2 The pink bollworm

1.2.1. The biology and life history of the pink bollworm

The pink bollworm, *Pectinophora gossypiella* (Lepidoptera:Gelechiidae) (Saunders), is one of the most destructive pests of cotton (*Gossypium hirsutum*) worldwide. Its main host is cotton, but it also feeds on wide variety of Malvaceae plants such as okra, kenaf and hibiscus.

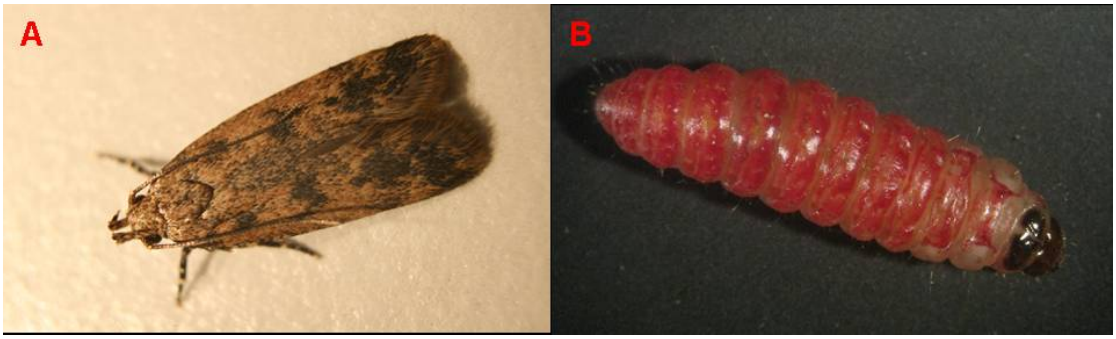

Figure 1.2. The pink bollworm. (A) Adult, (B) late larva.

The pink bollworm has four stages of development: egg, larva, pupa and adult, a small brown inconspicuous moth with a wingspan of 15-20 mm. The life cycle begins in spring when female moth lay eggs, mainly on the new shoots, flower buds and young bolls of the cotton plant. A female moth can lay 100-200 eggs. After hatching, first instar larvae immediately bore into the flower buds and cotton bolls where they feed on the seed kernels. Fully grown larvae emerge from the flower buds and cotton bolls, and drop to the ground to pupate. Adults are active only at night and the female moths produce a sex pheromone to attract the males for mating (Figure 1.2). In ideal conditions, the life cycle lasts about one month and there can be up to six generations per year (Hammad & Salam, 1985; Metcalff, 1993; Ellsworth et al., 2003).

Some larva remain inside the seeds or bolls left in the field or move into the soil, where they pupate, to overwinter and emerge next spring, or may give rise to a partial generation (EPPO Bulletin, 2004). Pink bollworm populations generally reach economically relevant densities late in the growing season. Early harvest of cotton is therefore another way to avoid pink bollworm outbreaks and to reduce overwintering populations (Carrière et al., 2001).

1.2.2. Origin and distribution of the pink bollworm

The origin of the Pink bollworm remains uncertain but it is believed to be of African origin based on comparison with closely related African species (Marlat,t 1918). More recently, it was suggested it is indigenous to Australia (Flint et al., 1975). Since being first reported in India around 1840 (Naranjo et al., 2002), the pink bollworm spread geographically throughout Asia (Marlatt 1918), North Africa (Ballou, 1920), and China (Hunter, 1918). Around 1913, it became established in Brazil, introducing the pink bollworm into the western hemisphere (Naranjo et al, 2002). This moth now inhabits many parts of the world including the Middle East, China, Brazil and the USA. Survival of the larva (in diapause) inside the seeds of the host plant is regarded as the main cause for its widespread distribution.

1.2.3. Damage caused by the pink bollworm

Pink bollworm damage is caused in various ways: primary damage by direct feeding of the larvae, this causes the attacked young bolls to fall off prematurely, secondary damage by bacteria and fungi. Secondary damage is aggravated in rainy years or in crops that receive overhead irrigation (EPPO Bulletin, 2004). The damaged cotton-

seed gives a lower ginning percentage, lower oil extraction and inferior spinning quality (poor quality lint). In Egypt, China and Brazil pink bollworm commonly causes cotton losses of 20%, and sometimes more (www.aphis.usda.gov).

The greatest damage carried out by the pink bollworm is to the lint, seed, yield and quality. The infested bolls are generally immature, have poor lint characteristics, and contribute little or nothing to yield. Burrowing activity stains lint, destroys fibres and reduces seed weight, vitality and oil content. The damaged seed-cotton gives a lower ginning percentage, lower oil extraction and inferior spinning quality. In 1996, the loss of yield in San Diego reached 25-35% (Reynolds & Leigh, 1967).

1.2.4. Pink bollworm control

Pink bollworm control is difficult because the larva, the life stage that damages the cotton, spends its development inside the cotton boll where it is inaccessible to both externally applied insecticides and natural enemies. The National Cotton Council estimates pink bollworm costs US cotton producers an estimated $21.6 million annually for prevention, control and yield losses (http://www.cotton.org/tech/pest/bollworm/upload/Pink-Bollworm-Eradication-A-Window-of-Opportunity.pdf).

1.2.4.1. Chemical control

Cotton uses approximately 25% of the world´s insecticides and more than 10% of pesticides (including herbicides, insecticides, and defoliants) (Allen Woodburn associates Ltd/ managing resources “Cotton: the crop and its agrochemicals market” 1995). In the USA, losses in the Imperial Valley (California) ranged from 8% to 79%

of the crop value from 1966 to 1980. The mean cost of insecticides used in the Imperial Valley during 1978-88 rose to a high of $640 per ha per year (Henneberry, 2002).

In the past, pink bollworm control relied mainly on chemical insecticides such as carbamates, organophosphorus compounds (OP), synthetic pyrethroids and spinosyn. In the US, the average number of applications is 6-7 per season (Cattaneo et al., 2006) but can be as high as 15 applications (Henneberry, 2002). Resistance to DDT occurred in Mexico in the late 1950s after 11-12 years of selection pressure (Lowry & Berger, 1965). By the mid-1980s there were reports of reduced effectiveness of some organophosphate and carbamate insecticides (Henneberry, 2002) and tolerance to pyrethroid insecticides (Henneberry, 2002; Haynes et al, 1986).

Development of insecticide resistance, high cost of insecticides, inefficiency of insecticides in killing larvae (due to their internal feeding habits), and the adverse effect of chemicals to populations of natural enemies and other wild life, render conventional chemical control an ineffective long-term solution to the pink bollworm problem.

1.2.4.2. Sexual confusion technique

Mating disruption works by interfering with the communication between male and female moths. Males distracted by an attractant that mimics the female pheromones, become disorientated and cannot find female mates, reducing the frequency of mating (Knipling & Mcguire, 1966; Grefenstette et al., 2008).

The first product, based on $C_{12\text{-}18}$ alkenol acetates, used to mimic sex attractant emitted by females was “Hexalure” (Green et al., 1969). In 1973, Hummel and

colleagues identified the natural sex pheromone of pink bollworm, a 1:1 mixture of the Z,Z- and Z,E-isomers of 7,11-hexadecadienyl acetate, and named it "gossyplure" (Lykouressis et al., 2005; Hummel et al., 1973). Since then other products, based on gossyplure, have been developed (Brooks et al., 1977, Henneberry et al., 1981; Flint et al., 1975; Staten et al., 1987). The results of several field trials demonstrate a substantial reduction in boll infestations and in the need for chemical insecticides when gossyplure is employed (Henneberry et al., 1981; Staten et al., 1995; El-Lissy et al., 1993). Gossyplure-based systems can also be used in conjunction with pesticides and do not appear to be a threat to insect predators (Grefenstette, 2008). Traps baited with gossyplure and placed in the cotton fields (Leggett et al,. 1994), at canopy level (Qureshi et al., 1993), have been shown to be an excellent tool for detection and monitoring of the pest population and is now an integral part of pheromone behavioural control (Shorey et al., 1976).

1.2.4.3. Cultural control

Cultural practices such as ploughing are important in reducing overwintering populations (Watson, 1980). Adkisoon et al. (1960) reported that cultural practices reduce moth emergence by over 80%. The removal and destruction of remaining cotton bolls also reduces the overwintering population (Kittock et al., 1973; Grefenstette et al., 2008).

1.2.4.4 Biological control

Large numbers of parasitoids and predators are reported to attack pink bollworm but, in general, the pink bollworm does not have effective natural enemies in cotton fields

(King & Powell, 1992; Malik, 2001). Efforts have been made to import and establish effective exotic natural enemies of this pest in cotton producing areas but they have proven unsuccessful (King & Powel, 1992).

Among egg parasitoids, species from the genus Trichogramma and Trichogrammatoidea are the most widely used for pink bollworm control. Malik (2001) reported that two applications of *Trichogrammatoidea bactrae* in cotton fields caused a total parasitisation in pink bollworm eggs of 19.6% and 26.8%, respectively, a low percentage of infestation. In another attempt at using biological agents against pink bollworm, *Chelonus sp. nr. curvimaculatus* (Cameron), an eggs-larvae parasitoid from Australia, was introduced into Colorado and Arizona. Although infestations of pink bollworm reached 69.6% on the year of the biological control agent release, its establishment was not achieved (Hentz et al., 1997). In 1972, *Chelonus blackburni* and *Bracon kirkpatricki* were released in cotton fields in Arizona (King & Powell, 1992) but infection rates only ranged from 9% to 25% (Bryan et al., 1976). Such low rates were insufficient to control pink bollworm.

In Australia, *Apantales* nr. *oenone* Nixon (Hymenoptera:Braconidae) and *Brachymeria pomonae* (Cameron) attack the larvae and pupae of pink bollworm, respectively. *B. pomonae* aggressively attacks the pupae of pink bollwrom but also the pupae of *A. oenone*. Due to this occurrence of hyperparasitism no attempts have been made to use *B. pomonae* as a control agent (White et al., 1998).

Although the potential of biological control has been studied, it remains difficult: the bio-ecology of most biocontrol agents is still relatively unknown and the inconspicuous larval feeding behaviour makes them inaccessible to natural enemies.

1.2.4.5. Pest-resistant cotton

Transgenic crop plants that produce insecticidal proteins of the bacterium *Bacillus thuringiensis kurstaki* (Berliner), simply known as *Bt*, have been engineered for pest control. *B. thuringiensis* is a gram-positive spore-forming soil bacterium that produces crystalline inclusions composed of proteins known as δ-endotoxins [Crystal (Cry) and Cytolytic (Cyt)] (Soberón et al,. 2007). These crystals are toxic to various insects from the orders Lepidoptera, Coleoptera, Hymenoptera and Diptera, and also to nematodes, with different isolates or variants having different species specificity. A major advantage of these toxins is their relative selectivity; therefore harmless to beneficial insects. These proteins are also highly biodegradable, they break down rapidly in the environment, and are innocuous to humans, vertebrates and plants (Bravo et al., 2007). In transgenic plants the Cry toxin is produced continuously, thereby maintaining toxin presence despite degradation; expression in appropriate tissues leads to exposure of chewing and boring insects to the toxin(s) (Bravo et al., 2007).

The first *Bt* cotton was commercially available in 1996 (Perlak et al, 1996). By 2002, transgenic cotton has been commercialised in eight countries: the USA, China, Argentina, Australia, India, Indonesia, Mexico and South Africa (Chaudhry, 2002). In 2001, roughly 4 million hectares of cotton were planted with *Bt* cotton. The USA accounted for about 60% of world *Bt* cotton acreage with nearly 2.4 million hectares planted, followed by China (37.5%) and Australia (2.5%) (Wrona et al., 1997; Frisvold et al., 2006; Grefenstette et al,. 2008; Patin et al., 1999, Henneberry, 2002). The development of *Bt* cotton was a major advancement in pink bollworm control. In the USA, it has been estimated that pest control costs have been reduced by $62-136

per hectare (Henneberry, 2002), with annual gains averaging $6-10 million (Henneberry 2002; http://ag.arizona.edu/arec/ext/btcotton/display.html).

Despite the outstanding success of *Bt* cotton in managing pink bollworm, a major threat to its use is the potential appearance of insect resistance. In the laboratory, several species of moths, including pink bollworm, mosquitoes and nematodes have developed resistance to the *Bt* toxins. Laboratory studies suggest that pink bollworm has the potential to increase its resistance to Cry1Ac toxin from a barely detectable level to over 100-fold in a single growing season (Simmons et al., 1998, Patin et al. 1999, Tabashnik et al., 2000), and that selection of highly resistant strains can be achieved in four generations (Henneberry, 2002).

Numerous efforts have been made in order to delay and prevent the development of *Bt* resistance in pink bollworm. These include the high dose/refugia strategy (Henneberry, 2002; Liu et al., 2001) and the use of cotton plants that express more than one *Bt* toxin (Tabashnik et al., 2000). Despite early concerns regarding potential development of pink bollworm resistance to *Bt* cotton, it continues to provide a high degree of control of pink bollworm; this might indicate that the efforts to prevent resistance development have been effective so far (Grefenstette et al., 2008).

1.3. The sterile insect technique

The sterile insect technique (SIT) is an insect population control method that relies on the release of sterile insects of a target pest over an affected region (Dyck et al., 2005). The aim is to dilute the native population's reproductive capacity, ideally to a level that leads to extinction of the native population (Atzeni et al., 1992). Insects of the target species are mass reared and are sterilised by ionising radiation. After being

released into the wild, the sterile males mate with wild females, which do not yield offspring, and minimise the possibility of wild males and wild females mating together to produce viable eggs (Gurr & Kvedaras, 2009). As a result the population will decline and over a sufficient period of time the target population will be controlled or even locally eradicated (Alphey & Andreasen, 2002). The sterile insect technique was the first involving insect genetics for population control, and it can be applied only to pest species that reproduce by sexual means.

1.3.1. Advantages and disadvantages of SIT

SIT has some advantages over other control strategies: it causes no environmental contamination as it relies on sterile insects rather than chemical or biological toxins. SIT does not leave harmful residues unlike most insecticides, it is species-specific, it uses the species' own mate-seeking ability, it has no negative impacts on non-target species, insects can be released immediately after irradiation and it does not introduce fertile exotic species into an ecosystem.

As well as these advantages, SIT has some drawbacks. It is an expensive technique, especially when used against dense or widely dispersed pest populations (Gurr & Kvedaras, 2009), and to achieve eradication a ratio of not less than 10:1 sterile to indigenous individuals should be released. Mass rearing facilities are expensive to maintain and there are some concerns regarding exposure of workers to radiation.

Sterilised males must be vigorous competitors with wild males for obtaining mates but mass rearing and irradiation can cause deterioration in the vigour of sterilised males, which can cause a 4-10-fold decrease in fitness (Alphey, 2002). In mass rearing high numbers of insects reared on a continuous basis provides the opportunity

for extremely rare genetic/molecular events to occur and the highly selective rearing conditions will ensure that the new traits will remain in the population. This may eventually make a strain unsuitable for release, either through reduced similarity to the target population or through loss of the characteristics for which the strain was bred. The use of dyes to mark the released insects, incorrect transportation of the strain from the laboratory to the infested area, and aircraft release can also have a negative impact on the performance of the insect to be released (Robinson, 2004).

Irradiation is generally carried out using gamma rays, which damage the chromosomes, induce dominant lethal mutations in reproductive cells (Kumano et al., 2008a). Irradiated insects may have reduced competitive mating ability compared to wild-type insects (Kumano et al., 2008a,b), have shorter life spans (Shelly et al., 1994; Alphey, 2002), reduced ability to transfer sperm (Barry et al., 2003, Seo et al., 1990; Taylor & Duval, 1999; Taylor et al., 2001) and reduced flight ability (Walder & Calkins, 1993). Another problem with SIT is that, in some cases, it is not desirable to release females. Despite being sterile, females can still cause damage. They may attempt to lay eggs or, in the case of mosquitoes, attempt to obtain a blood meal (Alphey & Andreasen, 2002), making them a nuisance to humans. It has also been shown that, for the Mediterranean fruit fly (medfly, *Ceratitis capitata*), releasing only males is more efficient then releasing both sexes (Rendon & McInnis, 2000). 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 (Alphey, 2002).

1.3.2 Use of SIT worldwide

The idea that pest insects could be controlled and eradicated through the use of sexually sterile males was conceived by Knipling in 1955 while working on the screwworm fly. Since then, this technique as been used worldwide to eradicate, manage or prevent establishment of several insect species.

One of the first successful cases of eradication achieved by SIT was the eradication of the new world screwworm (NWS), *Cochliomyia hominivorax*, an insect parasite of livestock and humans, in the 1950s in Curaçao. Eradication was achieved in only 7 weeks (Lindquist, 1955). Soon after eradication programmes started in the USA and Mexico and expanded to all of Central America. The USA and Central America are now free of NWS and re-invasion is prevented by a combination of quarantines and mass release of sterile NWS (Hoy, 2003). In the USA the estimated annual loss by the livestock industry was $20 million. The cost of eliminating this serious pest in that area was $8 million (Dame, 1984). In the late 80´s, the NWS was accidentally introduced into Libya. An eradication plan using SIT was put in place. Sterile fly releases were made over the infested area twice weekly and 500-1200 flies/km^2 (of both sexes) were released. Three years later, the NWS was considered eliminated (Vargas-Teran et al. 1994).

Perhaps the most successful and widespread application to date has been in the control of tropical fruit flies (family Tephritidae). In 1919 the melon fly, *Bactrocera cucurbitae*, was introduced into the Yaeyama Islands in Japan. It is a serious pest of the cucurbits, other fruit-crops and tropical fruit trees, and after its introduction it spread to other areas in Japan. In 1972, serious efforts were made to eradicate this pest by means of the sterile insect technique (SIT). A total of 78,348 million sterile flies

were released over an area of 4,504 ha and the total cost of the programme was $177.2 million. But by 1993, the pest was completely eradicated (Itô et al., 2003). The first SIT against the medfly, a major pest of fruit in many parts of the world, was initiated in Mexico in 1977 (Hendrichs et al., 1983). In 1982, the medfly was eradicated from Southern Mexico. The success of the Mexican project prompted several such projects in Central and South America; for instance, in Chile (Hendrichs et al., 2002) and in Argentina (Delongo et al., 2000). Most recently, in 1997, Israel and Jordan started the Arava Medfly Eradication Project (AMEP) in 1997. It serves as a pilot effort to begin implementing SIT in the Near East (Rossler et al., 2000). Another successful SIT programme was the eradication of the tsetse fly, *Glossina austeni*, from Unguja Island in Zanzibar in 1997 (Vreysen et al., 2000). A different goal is sought in Mexican fruit fly (*Anastrepha ludens*) programmes in southern California, where releases of sterile insects are conducted to prevent the establishment of these pests in uninfested, productive agricultural areas (Dame, 1984).

Among the Lepidoptera, SIT programmes have been carried out against the codling moth, *Cydia pomonella*, a serious pest of fruit, and the pink bollworm. A two-year long pilot programme was carried against the codling moth in Canada, in the late 70´s, resulting in marked reduction in the wild-type population (Proverbs et al., 1962). Following this success, in 1994, a full-scale programme to eradicate the codling moth was initiated in the Okanagan Valley, Canada. Although, the native codling moth population was reduced, eradication was not achieved (Judd & Gardiner, 2005).

A successful and ongoing SIT programme involves the pink bollworm in the San Joaquin Valley of California, USA, which as been in place since 1968 (Staten et al., 1993). This programme involves extensive surveillance using pheromone traps, use of transgenic *Bt* cotton, mating disruption and sterile pink bollworm moth releases.

Sterile pink bollworm adults are produced in a mass rearing facility in Phoenix, Arizona, and are sexually sterilised with gamma radiation (200 Gy from ^{60}Co) (Staten et al., 1995). Sterile pink bollworms are released every day, by aircraft, during the cotton-growing season, on approximately 1 million acres of cotton (Grefenstette et al., 2008). These efforts have proven successful in preventing the high populations of pink bollworm occurring in the adjacent regions of southern California, Arizona and northern Mexico, and in preventing the establishment of pink bollworm in the San Joaquin Valley (Staten et al., 1995). It has been estimated the programme has saved $175-200 per hectare in pest control costs (Dyck et al., 2005).

Several studies to test the feasibility of SIT use to control diamondback moth have been performed (Hudaya, 1976, 1983; Omar & Mamat, 1997), including studies on the effect of radiation on the moths (Hudaya, 1976, 1983; Bahari, 1994) and F_1 sterility (in which the progeny of moths irradiated with a lower radiation dose are sterile) (Thi & Than, 2001). A small-scale field trial was carried out by Hudaya (1976, 1983). He showed a dose of 300 Gy applied to pupae was able to induce sterility in adults without affecting adult emergence. After being irradiated these moths were released in cages with plants infested with diamondback moth at a rate of 9:1 (sterile:wild-type). Results show the reduction of the diamondback moth population by 61% in the next generation. Currently, no diamondback moth SIT programme is in place but some studies are in progress in Mauritius. However, it would still require considerable additional research to become operational.

1.3.3. Inherited sterility in Lepidoptera

Lepidoptera are more radiation-resistant than other insects. Studies show that most Dipteran, Hymenopteran and Coleopteran species could be sterilised with doses of radiation below 100 Gy whereas lepidopteran species require radiation doses of 200-400 Gy. Large differences in radiation-sensitivity between insect species belonging to different orders could be attributed to: (1) differences in radiation-repair mechanisms, (2) differences in oxygen concentration in the sperm and (3) holokinetic versus monokinetic chromosomes (LaChance & Graham, 1984).

Koval (1982) suggested that Lepidoptera are radiation-resistant because of 'superior excision-repair mechanisms´. LaChance & Graham (1984), in their work, discuss the remaining reasons for radiation-resistance of the Lepidoptera. They mention plant and animal cells are more sensitive to radiation when treated in oxygen and less sensitive when treated under anoxic conditions. Studies with the Caribbean fruit fly, *A. suspensa*, showed that decreases in the oxygen supply to the sample to be irradiated reduce undesirable effects, and that irradiation in nitrogen had fewer deleterious effects on the overall performance of the fly (Walder & Calkins, 1993). In Lepidoptera, the sperm remain in tight bundles until they are transferred to the female. In Diptera, loose individual sperm are stored in the basal portion of the testes or in the ejaculatory duct until mating occurs. If the bundles of sperm in Lepidoptera are comparatively anoxic, then this would confer a degree of radiation-resistance. The chromosomes of Lepidopteran species have diffuse centromeres (holokinetic chromosomes) (Marec et al., 2005). At one time it was thought that holokinetic chromosomes were responsible for the radiation-resistance of Lepidopteran species. However, Hemipteran and Homopteran species also possess chromosomes with

diffuse centromeres yet the milkweed bug and the lygus bug are not radiation-resistant (LaChance & Graham, 1984).

In 1962, Proverbs noticed that the F_1 progeny of irradiated male codling moths mated to non-irradiated females were sterile. Further studies (Amoako-Atta et al., 1979; North & Holt, 1970; Proshold & Bartell, 1973; Gonen & Calderon, 1971) showed that more F_1 male moths were produced than F_1 female moths (sex ratio distortion), F_1 progeny of irradiated male parents were more sterile than their fathers, and progeny of irradiated female parents are more fertile than F_1 progeny of irradiated male parents. Thus it has been suggested that the release of male Lepidoptera exposed to partially sterilising doses of gamma radiation would yield subsequent generation lines more sterile than the irradiated parents, thereby generating a built-in sterility factor in the population (Amoako-Atta et al., 1979). Male insects that are released are not in themselves "sterile"; they produce fully functional gametes that succeed in fertilisation and initiating development. The sterility is only apparent in the next generation; i.e. when the fertilised egg fails to complete development (Robinson, 2004).

F_1 sterility in Lepidoptera could be a potential tool for future control programmes as it would allow the use of lower radiation doses to induce partial sterility, producing better-quality, partially sterile moths with increased competitiveness compared to fully sterile moths. As lepidopteran females are generally more sensitive to radiation than males of the same species, radiation doses could be adjusted so that females are fully sterile and males are partially sterile (Robinson 2004). However, F_1 progeny are never completely sterile, meaning larvae, the damaging stage, would still be produced. Although lower doses of radiation would be used, irradiated insects are still not as competitive as non-irradiated insects (Carpenter et al., 1989; Proshold et al., 1993).

These constraints would have to be mitigated or bypassed before a F_1 sterility SIT programme could be implemented against lepidopteran species.

1.4. Insect transgenesis

Transgenesis involves the introduction of a segment of DNA, termed transgene, into an organism's genome to result in germ-line transmission (Venken & Bellen, 2007). Genetic transformation systems are a powerful tool for investigating gene function in several insect species (Kawashima et al., 2007; Robinson et al., 2004); providing new strategies for insect pest management (including improvements in SIT); impairing the transmission of pathogens by human and livestock disease vectors (Wimmer, 2003); and enhancement of beneficial insects (Hoy, 2003; Kramer, 2004; Wimmer, 2003).

In 1982, Rubin and Spradling described the first successful transformation of an insect, the vinegar fly *Drosophila melanogaster* Meigen (Diptera:Drosophilidae), by insertion of a wild-type copy of a defective eye-colour gene using a *P*-element-based vector. Subsequently, numerous attempts were made to transform other insects using the transposable *P*-element but without reproducible success (O'Brochta & Handler, 1988; Handler & O'Brochta, 1991; Handler et al., 1993; Hoy, 2003).

In order to transform non-drosophilid insects, other transposable elements were identified and in 1995, the first non-drosophilid insect transformation was described. Loukeris and colleagues (1995) successfully transformed the medfly using the transposable element *Minos*. Since then, other transposable elements have been identified and a much wider range of insect species transformed.

1.4.1. Transposable elements

Insect transgenesis has been achieved through the use of transposable elements. Transposable elements (TEs), or transposons, are DNA sequences that promote recombination reactions that result in their movement to new sites in the genome (O´Brochta & Atkinson, 1996). TEs are common and widespread, for instance, they comprise at least 15% of the genome of *Anopheles gambiae* (Sinkins & Gould, 2006) and over 32% of the human genome (Yoder et al., 1997).

TEs are classified in two classes based on their transposition intermediate and transposition mechanisms (Finnegan, 1992; Gbadegesin & Beeching, 2011): Class I and Class II. Class I TEs (retrotransposons) move by and amplify via RNA intermediates. The transposon is transcribed into RNA and then back to DNA by reverse transcriptase before being inserted into a new genomic location. Class II transposons transpose directly from DNA to DNA, using a copy-paste mechanism. The transposon is excised from its location and re-inserted into a new genomic location. This process requires an enzyme called transposase. All of the TEs commonly used in insect transformation, such as *P*, *piggyBac*, *Hermes*, *Minos*, *hobo* and *mariner*, are Class II TEs.

1.4.1.1. The *piggyBac* transposable element

The transposable element *piggyBac* was originally isolated from the genome of a baclovirus isolated from cell lines of the cabbage looper moth, *Trichoplusia ni* Hübner (Lepidoptera:Noctuidae) (Fraser et al., 1983, 1995). Structurally, it consists of a 2.1-kb unit, with a single open reading frame of 1.8 kb, flanked by two 13-bp perfect inverted terminal repeats (ITRs) and 19 bp subterminal repeats located 3 bp from the

5′ ITR and 31 bp from the 3′ ITR (Figure 1.3). The ORF encodes a functional transposase (Elick et al., 1996).

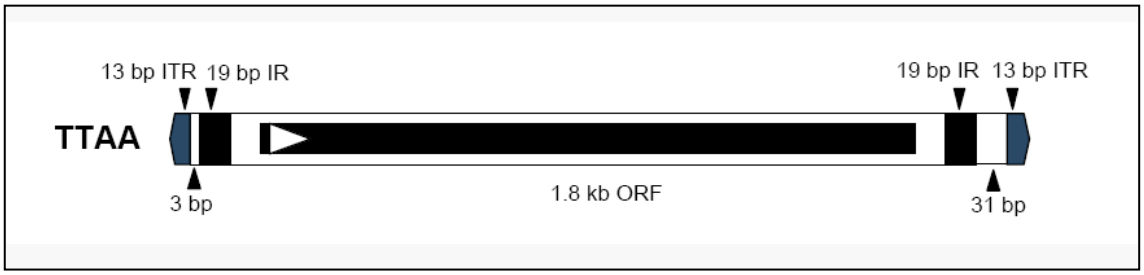

Figure 1.3. Diagram of the 2472 bp *piggyBac* element (from Handler, 2002). ITR: inverted terminal repeats sequences; IR: sub-terminal inverted repeat sequences; ORF: open reading frame; TTAA: tetranucleotide (TTAA) insertion site.

piggyBac transposition is accomplished when the transposase excises the transposons, by promoting double-stranded breaks in the donor DNA, with a hairpin intermediate, and then covalently joins it to the target DNA (Mitra et al., 2008). *piggyBac* transposons insert exclusively into a TTAA target site (O´Brochta & Atkinson, 1996; Handler, 2002), restoring the donor site to its pre-transposon state, so newly inserted transposons are flanked by TTAA at each end (Mitra et al, 2008). The medfly was the first insect to be successfully germ-line transformed using *piggyBac* (Handler et al., 1998) and since then this TE has been used to successfully transform several other species (reviewed by Handler 2002, Morrison et al., 2010).

1.4.2. Binary vector/helper transposon transformation system

Although there are several methods of integrating foreign DNA into a genome, such as biolistics and electroporation, the only method used for transformation of insects is microinjection of a mixture of two plasmid DNAs into preblastoderm embryos. One

plasmid is termed the transformation vector and consists of a transgene to be inserted, including all its regulatory sequences plus a marker gene and its promoter, flanked by the transposons inverted terminal repeats (ITRs). The second plasmid, termed the helper plasmid, is the source of transposase. It consists of the coding sequence for the transposase driven by a constitutive or inducible promoter (Figure 1.4).

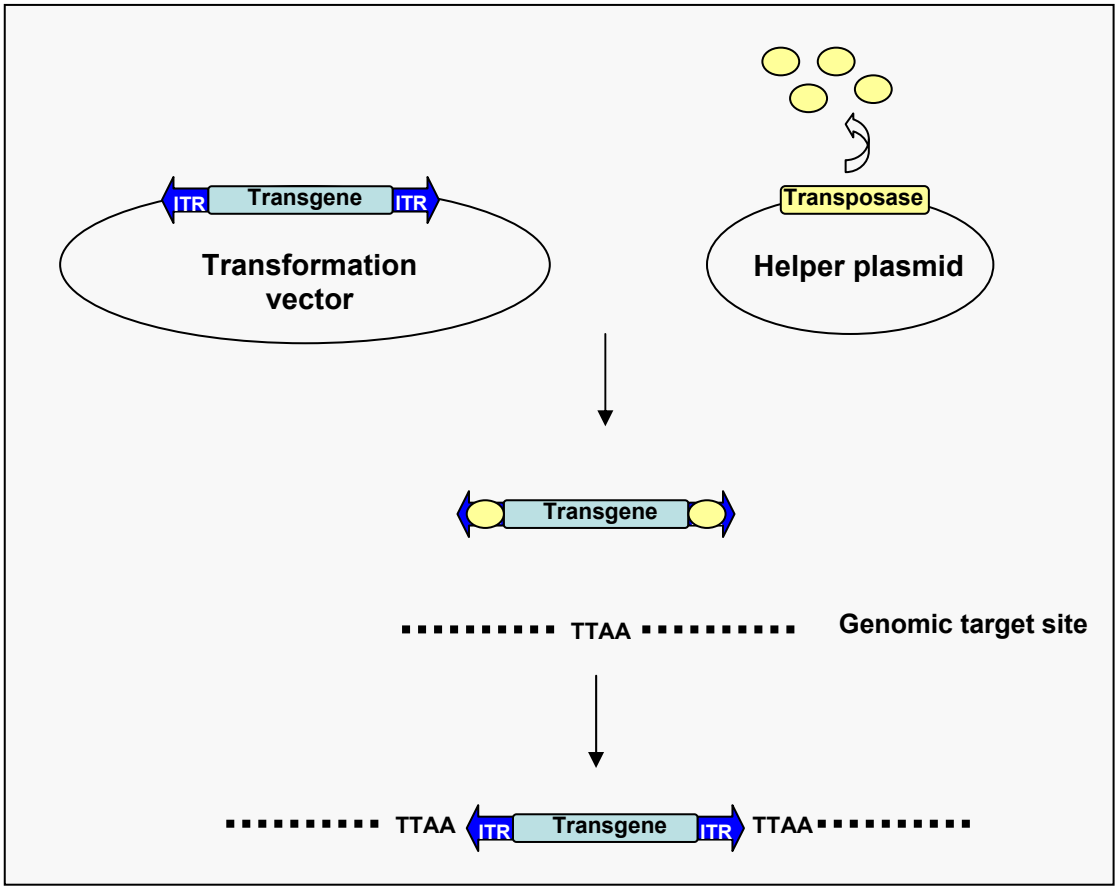

Figure 1.4. Binary vector-helper system based on the *piggyBac* transposable element. It involves the co-injection of two plasmids: a transformation vector carrying the transgene of interest and a non-integrating helper plasmid. The helper plasmid produces the transposase (yellow circle) which recognises its ITRs flanking the transgene cassette in the transformation vector. The transposase then catalyzes the instertion of the transgene into a cognate target site in the genome, TTAA.

When these two plasmids are co-injected, the transposase in the helper plasmid catalyses the movement of the donor cassette from the transformation vector into a

target site in the genome. If the vector were to be injected on its own, it would not be able to incorporate itself in the genome as it lacks the transposase gene. Similarly, if the helper alone were injected, it would not be able to insert itself because it lacks the inverted terminal repeats of the transposable element (Kramer, 2004), thus the ability to produce the transposase is lost with subsequent cell division and degradation of the helper plasmid. If integration occurs in somatic cells, the transgene will only affect the current generation. But if integration occurs in a germ-line cell, the transgene will likely be passed to the progeny of the individual, in a dominant fashion, in a Mendelian fashion. Assuming that host species does not have a similar endogenous TE, the transgene should remain stably inserted at its genomic site.

1.4.3. Marker genes

A critical component of a transformation system is the ability to separate transgenic from non-transgenic insects. Genes involved in the production of eye colour have been previously used as phenotypic transformation marker genes (Rubin & Spradling 1982; Kramer, 2004; Horn et al., 2002). In this mutant rescue selection method, the wild-type allele of a gene is introduced into a mutant strain, the wild-type allele complements the recessive mutation, and the visible phenotype is rescued (Horn et al., 2002). The use of eye colour genes as genetic markers allowed easy detection of transformed individuals, as mutations in these genes cause obvious phenotypes that are readily distinguished from the wild-type (Horn et al., 2002). This system worked well in *D. melanogaster* and was also used for the first transformations of medfly (Loukeris et al., 1995), the yellow fever mosquito (*Aedes aegypti*) (Jasinskiene et al., 1998) and the red flour beetle (*Tribolium castaneum*) (Lorenzen et al. 2002).

However, for most insect species neither visible mutants nor corresponding cloned genes are available. Appropriate mutants have to be selected and the corresponding gene cloned, which can be laborious. Furthermore, mutant strains are inbred and tend to be weaker than wild-type strains (Horn et al., 2002).

Genes that confer antibiotic resistance have also been used as selectable transformation markers. Several resistance markers have been used successfully in *D. melanogaster,* as listed in Horn et al. (2002): *neo* (Steller & Pirrotta, 1985), *opd* encoding an organophosphate hydrolase (Benedict et al., 1995), or the *Resistance to dieldrin* (*Rdl*) gene (Stilwell et al., 1995). However, drug resistance genes can be problematic. The main problem is that the degree of resistance conferred is not all that high, so it may be difficult to find a discriminating dose, especially considering that, in transposon-based systems, the random insertion of the transgene means the resistance gene will express differently depending on where it was inserted ('position effect'). Further investment in the development of drug-resistance systems might increase the degree of resistance. In addition, there may be concerns about releasing drug-resistant insects into the environment.

More recently, fluorescent protein genes have been used as markers. The first fluorescent marker to be developed was the green fluorescent protein (GFP) gene isolated from the jellyfish *Aequorea victoria* (Conica:Aequoreidae) (Prasher et al., 1992). GFP has proved to be easily detected *in vivo* and functional in different tissues (Horn et al., 2002), making it a good tool with which to identify transgenic individuals. It has also been used in a wide range of organisms: nematodes, *Caenorhabditis elegans* (Chalfie et al., 1994), bacteria, *Escherichia coli*, mammals (Ikawa et al., 1999), several insects from different insect orders (reviewed in Horn *et al*. 2002) and plants (Wenck et al., 2003). However, wild-type GFP has a high

excitation coefficient at near UV wavelengths (Day & Davidson, 2009), which might damage live organisms. In order to overcome this limitation, a mutant variation of GFP has been developed, the enhanced GFP (EGFP) (Cormack et al., 1996; Yang et al., 1996). EGFP is more soluble, has a lower and less harmful excitation peak (488 nm) and is reported to fluoresce 35 times more than GFP (Horn et al., 2002).

Other GFP enhanced variants with wavelength-shifted fluorescence have been developed (reviewed in Day and Davidson, 2009). These include colour variants such as the enhanced blue (EBFP) (Patterson et al., 1997), the enhanced cyan ECFP, and the yellow fluorescent protein (EYFP). The EBFP has limited use due to its rapid photo-bleaching (Horn. et al., 2002) and low brightness compared to GFP. The ECFP is a more innocuous blue light but it is difficult to separate from EGFP and excitation with short wavelength blue light causes increased auto-fluorescence (Horn et al., 2002). The yellow fluorescent protein (EYFP), one of the brightest variants has been shown to be useful in insect transgenesis (Horn & Wimmer, 2000).

The red fluorescent protein, DsRed, is from the coral-like sea anemone of the Genus *Discosoma* (Matz et al., 1999). DsRed has about 23% amino acid sequence identity to GFP (Horn et al., 2002) and it has been successfully used in the transformation of *D. melanogaster* (Handler & Harell, 2001a; Handler et al., 2004), *Anopheles stephensi* (Nolan et al., 2002), *Ae. aegypti* (Smith et al., 2007) and *Bombyx mori* (Royer et al. 2005). Variants of DsRed have been developed such as DsRed1 and DsRed2. DsRed1 is a humanised variant that shows the same properties as DsRed. DsRed2, a more soluble, less toxic and fast maturing version of DsRed1 (Horn et al., 2002), has been used in transgenesis of the medfly (Gong et al., 2005) and *Ae. aegypti* (Nimmo et al., 2006).

Other fluorescent proteins available for insect transgenesis include: TurboGFP, ZsGreen, yellow fluorescent such as PhiYFP and ZsYellow, AmCyan, JRed and CopGFP. JRed, CopGFP and PhiYFP have been successfully used in the transformation of medfly (Dafa´alla et al., 2006).

1.4.4. Promoters

For marker genes to be expressed, they must be under the control of robust and suitable promoters. Different promoters have been used to drive the expression of marker genes in insects. Constitutive promoters induce marker expression in all tissues of the insect body, in all developmental stages, and are, therefore, very useful as they permit the easy selection of transgenics at any life stages. A commonly used constitutive promoter, is the *Drosophila actin 5C* promoter. This promoter has been used successfully in mosquitoes: *Ae. aegypti*, *A. stephensi*, and *Culex quinquefasciatus* (Atkinson & O´Brochta, 1992). But it has shown less promising results in the stable fly, *Stomoxys calcitrans* (O'Brochta et al., 2000) and sheep blowfly, *Lucilia cuprina* (Atkinson & O´Brochta, 1992). This illustrates its limited usefulness in different species. Other examples, of constitutive promoters are the *Drosophila* heat shock promoters that have been used in germline transformation experiments for a number of different insect species (reviewed by Ashburner, 1989), and the the *D. melanogaster polyubiquitin* promoter used to generate transformants from *D. melanogaster* (Handler & Harrell, 1999), the Caribbean fruit fly, *Anastrepha suspensa* (Handler & Harrell, 2001b), and *L. cuprina* (Heinrich et al., 2002).

Promoters used for non-*Drosophila* insects include the baculovirus *immediate early (ie)* gene promoter from the *Autographa californica* (AcMNPV) and *Orgyia pseudotsugata (OpMNPV)* (Pfeifer, 1998). Hr5*ie*1, a fragment of the *immediate-early*-1 (*ie*1) gene from *Ac*MNPV flanked by the Hr5 enhancer element, has been used in the transformation of the medfly (Gong et al., 2005) and *A. gambiae* (Grossman et al., 2001). *Opie2*, the *Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early-2 promoter as been used in silkworm cell culture assays (Nakayama et al., 2006). As both Hr5*ie1* and Opie2 have been shown to be active in different insect orders, they are potentially useful tools in insect transformation. The *B. mori* A3 cytoplasmic actin gene promoter (*BmA3*) was used in the first germline transformation of the silkworm (Tamura et al., 2000) and the pink bollworm (Peloquin et al., 2000). However, its maximal transcription is in microfilament-rich, non-muscular organs such as larval midgut and silk gland (Mounier & Prudhomme, 1991; Horn et al., 2002; Mange et al., 1997) - highly auto-fluorescent tissues - which could make fluorescence detection difficult.

Promoters that only active in one or few tissues or cell type also been used in insect transgenesis. The eye-specific artificial 3×P3 promoter consists of three tandem repeats of the P3, the binding site of Pax6, a transcriptional activator that controls the genetic pathway of eye development (Berghammer et al., 1999). It has been designed to drive expression in the eyes and nervous system of the larvae, pupae and adult insects. It is routinely used in the germline transformation of mosquito species. In lepidopteran species, it has been shown to be active in the silkworm (Dai et al., 2005) and the butterfly *Bacyclus anynana* (Marcus et al., 2004).

1.5. Release of insects with a dominant lethal (RIDL)

Conventional SIT programmes have proven to be successful. However, there are still some aspects that can be improved, such as, the replacement of radiation by another sterilising agent, in order to reduce the negative impacts of radiation on the released insects.

In 2000, Thomas and colleagues proposed a genetic version of SIT called Release of Insects with a Dominant Lethal (RIDL®). In such a system, all the insects to be released would be homozygous for a dominant lethal and would be reared under permissive conditions (repression of the lethal gene). After release, released insects would mate with wild-type insects and pass one copy of the dominant lethal gene to their offspring. As the permissive condition is not present in the wild, the offspring would die. A major advantage of this technique is that released insects do not need to be sterilised and, therefore, do not need to be irradiated.

Another advantage of RIDL is that lethality can be engineered to match specific species requirements (Labbé et al., 2010). Genetic sexing, which allows rapid and efficient separation of females and males, could be integrated in this system by using a female-specific dominant lethal (Alphey & Andreasen, 2002). As above, homozygous insects carrying a female-specific dominant lethal would be reared under a permissive condition. Prior to release, the permissive condition would be removed, killing the females and allowing only males to be released. The released males would mate with wild-type females and pass the female-specific dominant lethal to their progeny. Female offspring would die and male progeny would survive and pass the female-specific dominant lethal to half of their progeny. Only male release would be very useful in species such as mosquitoes, where the females bite, or fruit flies, where

the females cause damage by piercing the fruit for oviposition. Early lethality could also be engineered (Horn & Wimmer, 2003; Schetelig et al., 2009) and might be the ideal scenario for agricultural pests like the fruit flies and moths or screwworm in which larvae are the most damaging life stages.

1.5.1 Tetracycline-repressible system

RIDL relies on the repression of the dominant lethal gene by a repressible factor, such as temperature or a chemical. The best-characterised and most versatile conditional approach is the tetracycline-repressible system (Gossen & Bujard, 1992). The tetracycline-repressible system relies on the specific and high-affinity binding of the *E. coli* tetracycline repressor protein (TetR), or its derivatives, to the tetracycline operator (*tetO*) sequences (Stebbins et al. 2001). In 1992, Gossen & Bujard, fused the TetR with the herpes simplex virus VP16 activation domain, creating the tetracycline-responsive transactivator (tTA), and made this system applicable to eukaryotes.

When applied to RIDL, the tetracycline-repressible system was first delineated as two-component system (Figure 1.5). The first component consists of a promoter controlling the tTA expression. The second component consists of a lethal effector gene under the control of the tTA response element, *tetO*. In the absence of tetracycline, the tTA protein binds to *tetO*, activating the transcription of the *tetO*-driven lethal gene, leading to cell death. In the presence of tetracycline, the tTA binds preferentially to the tetracycline, preventing it to bind to *tetO* sites. As a result, transcription of the lethal effector gene is repressed, allowing the insects to develop normally on a diet supplemented with tetracycline. A simplified version of this system, the single-component positive feedback system (Figure 1.5) was developed

by Gong and colleagues (2005), where tTA is both the transactivator and the effector. In this system, the tTA coding region was placed under transcriptional control of a minimal promoter. In the absence of tetracycline, basal amounts of tTA, produced by the minimal promoter, bind to *tetO* leading to more synthesis of tTA. The accumulation of large amounts of tTA is deleterious to the cell due to transcriptional squelching (Berger et al., 1990), i.e., titration of transcription factors by the VP16 component of tTA resulting in reduced expression of unrelated genes (Stradthee et al., 1993; Matis et al., 2001).

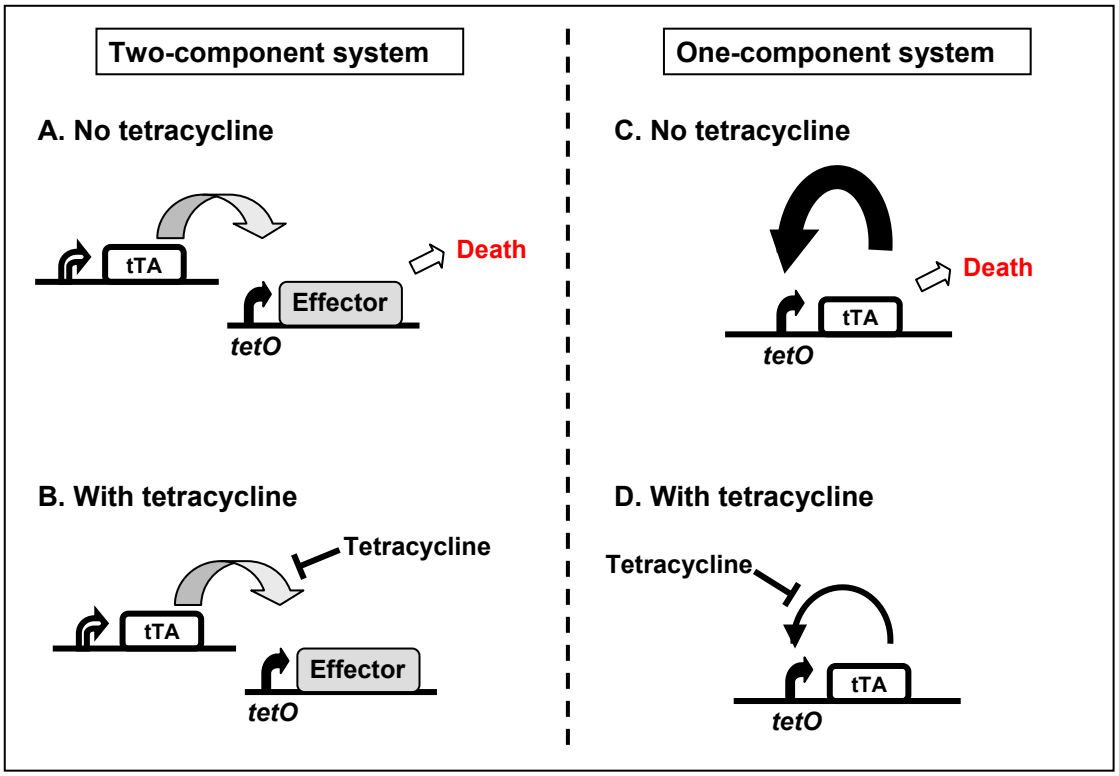

Figure 1.5. Tetracycline-repressible systems. In the two-compenent tetracycline repressible lethal system: (A) in the absence of tetracycline, expressed tTA binds to *tetO*, deriving expression of the lethal effector molecule, leading to death. (B) In the presence of tetracycline, tTA preferentially binds to tetracycline, the effector molecule is not expressed and there is no lethal effect. In the one-component positive feedback system (C) low levels of basally expressed tTA bind to *tetO*, driving further expression of tTA. When tTA levels become high, it becomes deleterious to the cell, acting as a lethal effector molecule, leading to death. (D) In the presence of tetracycline, tTA preferentially binds to tetracycline and the level of tTA remains basal. A low level of tTA is not lethal (Adpted from Gong et al., 2005).

The tetracycline-repressible system offers several advantages: responds to low concentrations of tetracycline, efficient on/off response, and works in many species such as the malaria mosquito *A. stephensi* (Lycett et al., 2004), *Ae. aegypti* (Fu et al., 2010), *D. melanogaster* (Stebbins et al., 2001), medfly, the nematode *C. elegans*, the plant *Nicotiana tabacum* and a human cell line (Koukidou et al., 2006). Furthermore, this system has been used with success in conditional embryonic lethal system, analogous to the one proposed here. Reproductive sterility based on the lethality of the progeny, conditional embryonic lethal systems, have been developed for *D. melanogaster* (Horn and Wimmer, 2003) and medfly (Schetelig et al., 2009), where the expression of a dominant lethal, driven by a promoter active in early embryo development resulted in non-viable embryos.

1.6. Research objectives

With the general aim of contributing to the field of pest insect control by genetic modification, and more specifically control of Lepidoptera, I have set out in my DPhil to

1) Establish a germline transformation system for diamondback moth using the *piggyBac* transposable element (Chapter 3)
2) Investigate the utility of the ΦC31-integrase and the Cre- and Flp-recombinases in site-specific manipulation of the diamondback moth genome (Chapter 4)
3) Investigate the use of maternally expressed gene promoters from *B. mori* in pink bollworm, and their potential use in the development of a tetracycline-repressible female sterile system in pink bollworm (Chapter 5)

Pink bollworm was selected as a target species due to its existing status as a target of SIT in south-western USA. This species has also been worked with for several years in my DPhil laboratory, and transformation is now almost a routine (if labour-intensive) exercise. It can therefore be regarded as a good candidate for development of such genetic tools in Lepidoptera, and one against which the proposed genetic control technology can realistically be used.

Diamondback moth, on the other hand, has not been previously transformed. However, if germline transformation could be accomplished, this achievement would permit the development of RIDL against diamondback moth and its potential use in SIT-type control programmes in the future. It may also open the way for the used of this species as a new model species in broader scientific research.

Chapter 2

Materials and Methods

2.1. General Insect Rearing

2.1.1. Diamondback moth stocks

Wild-type diamondback moths were cultured from a strain obtained from Syngenta laboratories, Jealotts Hill, UK. All life stages of diamondback moth were incubated at 27± (1ºC), 60% (±2%) RH and 12:8 light:dark. Pupae were placed in a 350×252×130 mm cage and, after emergence, adults were fed with a sugar water solution (a mixture of 100 µg/ml tetracycline, 7.5% sucrose and 0.07% methylhydroxybenzoate), by placing a piece of cotton wool impregnated with sugar water in the cage. Eggs were collected by placing inside the cage several Parafilm strips (60×150 mm), coated with cabbage extract applied with a paintbrush and air-dried. The adults were left to lay overnight and next day the Parafilm strips were moved to a 355 ml (602×246×549 mm) disposable plastic container (Bare^{TM} DeliGourmet, Solo Cup Company, USA) containing 100 ml of beet armyworm artificial diet (Bio-serv®, USA). The container was sealed with a layer of cardboard, a layer of mesh, which served as a pupation surface, and a perforated plastic lid. Larvae were left until they pupated. As many pupae as possible were then collected to start a new stock cage. All of the stock cages and instruments used to handle the insects and the food were autoclaved before being used, to prevent fungal growth.

2.1.2. Pink bollworm stocks

Insect stock colonies were set up with approximately 200 pink bollworm pupae sent to our laboratory, on a weekly base, from the APHIS mass-rearing facility in Phoenix, USA. Stock colony cages consisted of a 341-ml paper beaker (Ambicon UK Ltd, London, UK) with a netting lid. Adults were fed with sugar water, as above, by

placing a piece of cotton wool impregnated with sugar water on top of the netting. Stock cages were kept in a plastic box in order to maintain adequate humidity inside the stock cages and to avoid cross-contamination between stock and other strain cages. Stock cages were kept at 27±1ºC and 60% RH on a 16-h day/8-h night cycle. New stock cages were set up every week.

2.1.3. Silkworm stocks

Silkworm eggs, *Bombyx mori* (Dazao strain), were obtained from Warwick Insect Technologies Ltd (Wantage, UK). After eclosion, first instar larvae were transferred, with the help of a paint brush, to a plastic container 250×130×50 mm and kept at 25±1ºC and 60% RH on a 16-h-day/8-h-night cycle. Larvae were provided with a *Morus sp*.-based artificial diet, also obtained from Warwick Insect Technologies Ltd (Warwick, UK), every 2 days or when required. Pupae were removed from the cocoons and sexed. One female and one male pupa were paired in an open plastic container and kept in the same conditions as eggs and larvae.

2.2. Germline transformation

2.2.1. Preparation of injection mixes

2.2.1.1. Preparation of injection buffer stock

Injection buffer (1×) stock was prepared by adding the following ingredients: 1 ml of 0.1M Sodium phosphate buffer (46.3 ml of 1M Na_2 H_2PO_4 solution and 53.7 ml of 1M NaH_2PO_4 solution, pH 6.8.), 1 ml of 50 mM KCl solution and 7 ml of deionised water.

The injection buffer was then filter-sterilised, using individually wrapped Whatman 0.2 µm CA w/ GMF filter end and sealed 13 ml syringe (Whatman, USA), aliquoted and frozen at -20ºC.

2.2.1.2. Injection mixes with construct DNA – helper DNA

To prepare the DNA-DNA mixture, 500 ng of DNA construct and 350 ng of DNA helper, both prepared using a maxi-prep Kit (Qiagen, UK, see below), were diluted in 1× injection buffer. Deionised water was added up to 10 µl. Mixes were stored at -20ºC.

2.2.1.3. Injection mixes of construct DNA-mRNA helper

To prepare DNA-mRNA helper injection mixes, 500 ng of DNA (prepared as described in Chapter 2, section 2.4.1) and 350 ng of mRNA helper were diluted in 1× injection buffer (see Chapter 2, section 2.2.1.1). Deionised water was added up to 10 µl. Mixes were stored at -80ºC.

2.2.1.4. Injection mixes with mRNA only

To prepare injection mixes containing only mRNA, 700 ng of mRNA was diluted in 1× injection buffer (see Chapter 2, section 2.2.1.1). Deionised water was added up to 10 µl. Mixes were stored at -80ºC.

2.2.2. Embryo collection for microinjection

2.2.2.1. Diamondback moth

Diamondback moth egg-laying cages were set up and kept as wild-type stock cage as described in section 2.1.1 (Figure 2.1A) Embryos for microinjection were collected in a standard microscope slides placed inside the stock cage, during the light phase of light cycle. To encourage the adult moths to oviposit, cabbage extract was painted, using a fine paintbrush, onto the upper face of the standard microscope slide and allowed to air dry. Cabbage extract was prepared by placing a small piece (10 mm^2) of cabbage in an eppendorf tube to which a small volume of water was added (1 ml). A pestle was used to macerate the leaf and form a leaf suspension. Once dry, the slide was placed inside the cage and left until sufficient embryos were laid on the surface (approximately 200 embryos). Depending on how well the moths were laying, 10 to 30 minutes were usually sufficient to collect the desirable number of embryos. When the appropriate numbers of embryos were laid the slide was removed from the cage and the embryos ready to inject without the need of being sterilised, dried or the addition of oil. The slides collected for injection can be replaced with new ones for as long as the moths continue to lay.

2.2.2.2. Pink bollworm

Egg-laying cages were set up as described in section 2.1.2 and were kept at 27±1ºC and 60% RH on a 12-h day/12-h night cycle, inside an incubator. Since the pink bollworm mainly lays eggs in the dark, the dark phase of the light cycle should match the microinjection period. Egg-laying commenced 3-4 days after adult eclosion. Pink bollworm moth embryos were collected by placing a standard, clean glass microscope

slide onto the top of the egg–laying cage (Figure 2.1B). When the appropriate numbers of eggs were laid (200 approx) the slide was removed from the cage and ready to inject without the need of being sterilised, dried or the addition of oil. The slides collected for injection can be replaced with new ones for as long as the moths continue to lay.

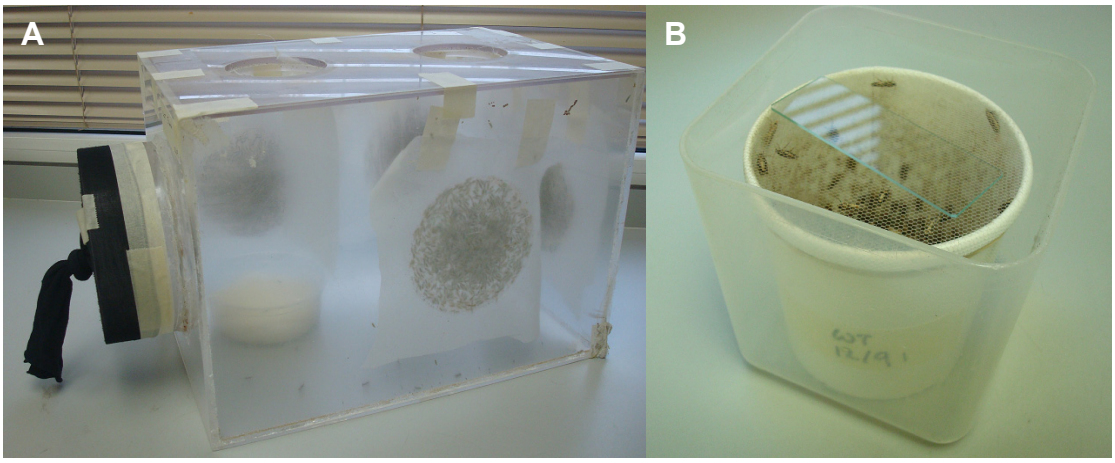

Figure 2.1. Egg-laying cages. Photograph showing a diamondback moth egg-laying cage (A) and a pink bollworm egg-laying cage with a glass slide on top to collect embryos for injection (B).

2.2.3. Microinjection

Diamnondback moth and pink bollworm transgenics were generated by microinjecting embryos using an Eppendorf Micromanipulator InjectMan connected to an Eppendorf FemtoJet® pressure modulator and ready-to-use Femtotip® II needls (Eppendorf, Germany). Embryos were viewed under an AE20/21 Motic Inverted Microscope (Motic®, USA).

Prior to injection all injection mixtures were centrifuged at 13,000 × *g* for 5 min to eliminate any particles that might block the needles. Using an Eppenford Microloader (Eppendorf, Germany), Femtotip® II needles were back-filled with 4µl of injection

mix and attached to the pressure supply of the micro-injector (Eppendorf FemtoJet® pressure modulator). The injection mix was injected into the posterior pole of the embryos where the germ line forms. In both species, the posterior end of the embryo is the smoothest and narrower end of the embryo while the anterior end is slightly 'ruffled' and not as rounded (Figure 2.2 and Figure 2.3). After piercing the embryo, a small volume of injection mix was injected into the embryo which could be detected by the movement of the embryo's contents as the liquid entered the embryo. The needle was then removed from the embryo and the next embryo was injected. Injections were performed with injection and compensation pressures maintained at 200hPa and 100hPa, respectively, and a 300 ms pulse. Injection pressure was adjusted as required, depending on the viscosity of the injection mix. Following injection, the number of eggs per slide was recorded.

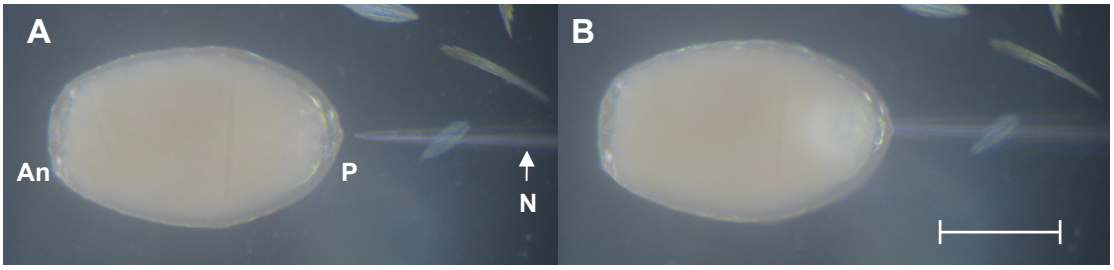

Figure 2.2. Pink bollworm embryo.Photograph of pink bollworm embryo, prior (A) and during (B) injection, indicating the posterior (P) and anterior (An) poles, as viewed through injection microscope, as well as the target site of injection and needle (N). Scale bar represents 0.2 mm.

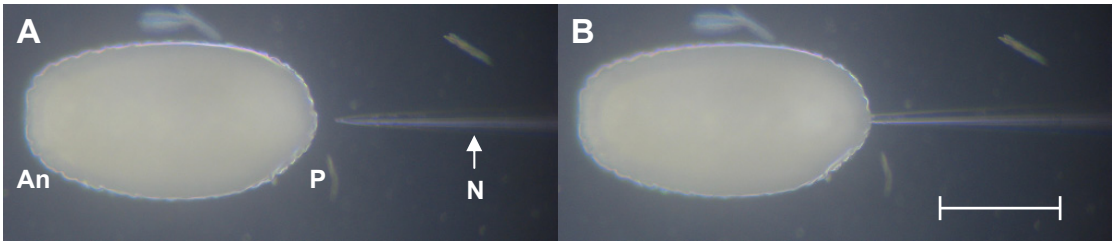

Figure 2.3. Diamondback moth embryo. Photograph of diamondback moth embryo, prior (A) and during (B) injection, indicating the posterior (P) and anterior (An) poles, as viewed through injection microscope, as well as the target site of injection and needle (N). Scale bar represents 0.2 mm.

2.2.4. Post-injection care

2.2.4.1. Diamondback moth

Slides containing injected embryos (G_0) were placed inside a plastic box with some damp cotton wool soaked in water. The embryos were left to recover for approximately 48 h at 27ºC after which they were transferred into a 250 ml (90 mm height) plastic beaker (Fisher Scientific, UK) containing 30 ml of beet armyworm artificial diet (Bio-serv®, USA) and covered with a layer of towelling paper with a glass lid on top. Eggs and larvae were left to develop inside the cup maintained at 27°C and 60% RH. Extra food was added if required.

2.2.4.2. Pink bollworm

After injection, G_0 embryos were placed inside a 90 mm Petri dish (Appleton Woods, UK) which was in turn placed inside a zip-lock plastic bag with some damp tissue. The embryos were kept in the dark, at 27°C and 60% RH, for approximately 3 days. When the embryos were nearly ready to hatch, artificial diet - obtained from the mass-rearing facility in Arizona USA - was added to the Petri dish. Larval development was checked regularly and when the fully grown larvae were about to pupate, the Petri dishes were removed from the zip-lock bag and moved to a large plastic box (325×235×145 mm) (Azpac Limited, Loughborough, UK) containing HexWeb® Honeycomb (Hexcel, provided by the Aphis mass rearing facility in Arizona, USA) which provide refugee for pupation.

2.2.5. Establishing transgenic lines

For both species, survivors of microinjection (G_0) that pupated were collected, sexed and crossed to wild-type mates.

2.2.5.1. Diamondback moth

G_0 pupae were collected, sexed (see appendix 1) and crossed to wild-type pupae of the opposite sex, in pools, in a ratio of 1:3 (♂:♀). The pupae for each cross were placed inside a 90 mm Petri dish painted with cabbage juice (to encourage oviposition on the bottom of the dish) and with a small piece of cotton wool saturated with sugar water. Every 2 days the eggs were collected by anaesthetising the adults with CO_2 and transferring them to a new Petri dish. Approximately 25 g of beet armyworm artificial

diet was added to the Petri dish, which was sealed with a square of towelling and a lid. G_1 progeny (the progeny of G_0) were screened for fluorescence from the transformation marker as pupae under an Olympus SZX12 stereomicroscope with the appropriate filters for each fluorescent protein [filters:RFP1 (red): exciter 520-550, emitter 580 , GFP (green): exciter 460-490, emitter 470-495, CFPA(cyan): exciter 400-440, emitter 470-495]. Transformants from each G_0 cross were then sexed and crossed, in pools, to wild-type mates and reared as G_0, establishing an independent transgenic line. Non-fluorescent G_1 were discarded.

2.2.5.2. Pink bollworm

Crosses were established based on a 1:3 (♂:♀) ratio. For each cross, pupae were sexed (see appendix 1) and placed in a paper cup, as describe in section 2.1.2. Embryos were collected by placing a piece of filter paper on top of the netting. Every 2 days the filter paper was removed and replaced. Collected filter paper, with the embryos, was placed inside a Petri dish and was covered with food supplied by the APHIS mass-rearing facility (Arizona, USA). The Petri dish lid was modified so that its central area was replaced with lidding paper, to allow the final-instar larvae to chew through in order to exit the Petri dish for pupation. The Petri dish was placed inside a plastic box with Hexcel where the larvae pupate. G_1 pupae were removed from the Hexcel and screened for fluorescence, as described above (section 2.2.5.1). Transformants from each G_0 cross were sexed and crossed to wild-type of the opposite sex, establishing an independent transgenic line. Non-fluorescent G_1 were discarded.

2.3. Photomicroscopy

All images were captured with an Olympus SZX12 stereomicroscope, fitted with a Canon Powershot S5IS digital camera.

2.4. Nucleic acid isolation

2.4.1. Plasmid DNA isolation

Small scale plasmid DNA preparation, up to 20 μg of plasmid DNA, was performed with the GeneJET Plasmid Miniprep Kit (Fermentas) following the manufacturer's instructions.

Medium- (up to 100 μg) and large-scale (up to 500 μg) plasmid DNA purification was performed using the QIAfilter Endofree plasmid Midi and Maxiprep kits (Qiagen, UK), respectively, following the manufacturer's instructions.

2.4.2. Preparation of genomic DNA

Genomic DNA preparations for utilisation in PCR reactions were performed using the GeneJET^{TM} Genomic DNA Purification Kit (Fermentas), according to the manufacturer's instructions.

2.4.3. Preparation of total RNA

Total RNA from pupae, adult moths or moth tissues were purified using TRI Reagent® Solution (Ambion®, USA), following manufacturer's instructions, and eluted in 30 µl of MilliQ water.

2.4.4. Preparation of mRNA for injection

mRNA for injection was transcribed and purified using the mMESSAGE mMACHINE® T7 Kit and MeEGAclear™ (Ambio®, Austin, USA) following manufacturer's instructions. The initial template DNA used was obtained by linearisation of plasmid DNA with a restriction enzyme downstream of the insert to be transcribed. The following ΦC31, Cre and FLP recombinase-based template DNA helper constructs were digested in a 100 µl reaction, at 37ºC in a dry incubator for 3-4 h, with the restriction enzymes listed in Table 1.

Table 2.1. Restriction enzymes in the preparation of mRNA used in microinjections. Maps of the constructs can be found in appendix 2.

DNA construct	Description	Restriction enzyme
OX3869	Wild-type ΦC31 integrase	*Bam*HI
OX4505	Codon optimised ΦC31	*Bam*HI
OX4533	Codon optimised ΦC31 integrase flanking by two nuclear localisation signals	*Bam*HI
OX4611	Wild-type *Cre* recombinase	*Xba*I
OX3608	Wild-type *FLP* recombinase	*Xba*I

2.4.5. Precipitation of nucleic acids

When it was necessary to concentrate or clean DNA, ethanol precipitation was carried out by the addition of 2.5 volumes of 100% ethanol, in the presence of 0.1 volume of 3M sodium acetate, pH 5.3, to samples. The precipitation was then allowed to occur at -20ºC for at least 1 h before DNA was pelleted by centrifugation for 30 min at 13,000 × *g*, at 4ºC. The DNA pellet was washed once with 500 µl 70% ethanol and re-suspended in an appropriate volume of TE buffer.

RNA precipitation was conducted as DNA precipitation except for the usage of 1 volume of isopropanol instead of 2.5 volumes of 100% ethanol.

2.4.6. Quantification of nucleic acids

DNA concentrations were estimated by spectrophotometry at 260 nm, using a GeneQuant II RNA/DNA Calculator (Amersham Pharmacia Biotech Inc., New York, USA). Readings were zeroed with the solution in which the samples had been diluted. The ratio of A_{260}/A_{280} provided an estimation of protein contamination. RNA concentration was estimated by running 0.5-1 µl of RNA in a 1% agarose gel with RiboRuler^{TM} High Range RNA Ladder (Fermentas, Vilnius, Lithuania), prepared following manufacture's instructions, and comparing its intensity to those of ladder bands if known amount of RNA the ladder under a UV illuminator.

2.5. Restriction digestions

DNA digestion was performed in a water bath using restriction endonucleases (New England Biolabs Inc., Ipswich, USA), at the recommended temperature and

concentrations of enzyme, buffer and BSA. Where double digests were performed, digests were either performed in a single reaction, or in sequential digests with a reaction clean up using MiniElute® Reaction Cleanup Kit (Qiagen, Crawley, UK) in between digests, depending on buffer compatibility. Where plasmid or insert was required for a ligation reaction, 0.5-1 µg of DNA was added to a total reaction volume of 20 or 30 µl. Diagnostic digests were performed in a 10 µl total volume with 0.1-0.2 µg of DNA.

2.6. De-phosphorylation of DNA fragments

Antartic Phosphatase (New England Biolabs) was used to remove 5' phosphates from vector DNA, according to the manufacturer's instruction, in order to prevent them from self-ligating thus decreasing the vector background in cloning strategies.

2.7. Phosphorylation of oligonucleotides

Addition of a phosphate group to the 5' end of single stranded oligonucleotides was performed with T4 Polynucleotide Kinase (PNK) enzyme (New England BioLabs). When phosphorylation was required prior to the annealing of complementary oligos, the 1× PNK buffer was replaced with 10×T4 DNA ligase buffer (New England Biolabs) as per the manufacturer's instructions. The T4 Ligase buffer provides the required ATP for annealing.

2.8. Agarose gel electrophoresis of nucleic acids

DNA and RNA were separated by electrophoresiss at 120 V in a 0.5-1% agarose gel [agarose and ethidium bromide, final concentration of 0.5 μg/ml, dissolved in 1× TAE buffer], using 1× Tris-acetate-EDTA (TAE) buffer [40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4] as the running buffer. DNA fragment size was determined by comparison with a DNA ladder, Smartladder (Eurogentec, Southampton, UK) for DNA fragments from 200 to 10,000 bp or to a 100 bp DNA ladder (New England BioLabs). RNA fragment size was determined by comparison with an RNA ladder, RiboRuler^{TM} High Range RNA Ladder (Fermentas). Prior to loading, 6× loading dye [0.25% (w/v) bromophenol blue and 30% (v/v) glycerol in H_2O] was added to DNA or RNA samples to a final 1× concentration.

2.9. Gel extraction

DNA was excised from the gel using a sterile scalpel under a UV illuminator and purified using the QIAquick Gel Extraction kit (Qiagen), following the manufacturer's instructions.

2.10. PCR purification of DNA

DNA was purified from enzymatic reactions using the QIAquick PCR purification kit (Qiagen), following manufacturer's instructions.

2.11. Ligation of DNA

Ligations were performed using the Rapid DNA Ligation Kit (Fermentas) at a molar ratio of 1:3 vector:insert, in a 20 µl reaction, following the manufacturer's instructions.

2.12. Molecular cloning of PCR products

PCR products were cloned into a pJET1.2/blunt vector molecule using the CloneJET^{TM} PCR Cloning Kit (Fermentas), according to manufacturer's instructions. The only variant in the protocol was the use of 'half volume' reactions, where 1 µl of PCR product was added to 9 µl reaction mix, to save on reagents.

2.13. Transformation into *E. coli*

To each tube of XL10-Gold® Ultracompetent Cells (Stratagene, Santa Clara, USA) cells, 4 µl of β-Mercaptoethanol was added, and then were incubated on ice for 10 mins. Between 20-30 µl of cells were added to 2-4 µl of ligation mix and incubated for 30 min on ice. Cells were heat-shocked in a 42ºC water bath for 30 s, and immediately transferred to ice where they were incubated for 2 min. A total of 200 µl of transformation medium [10mM $MgCl_2$, 10mM $MgSO_4$, 20% glucose, LB medium] was added, and the cells were incubated at 37ºC on a shacking incubator at 250 rpm for at least 1 h. Using a Bunsen burner and sterilised spreader, cells were plated at a variety of volumes on pre-warmed agar plates containing an appropriate concentration and type of selective antibiotic. When required, for blue/white colony selection, 40 µl

of IPTG (isopropyl β-D-1-thiogalactopyranoside) and 40 µl of 40 mg/ml X-GAL (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) dissolved in dimethylformamide (DMF) were added to the L-agar plate 1 h prior to spreading cells. Plates were incubated at 37ºC overnight.

2.13.1. Antibiotic usage

Selection for ampicillin resistance on L-agar or in L-broth was performed at 100 µg/ml, from a 100 mg/ml stock solution (w/v) in 50% water and 50% ethanol. Selection for kanamycin resistance L-agar or L-broth was performed at 50 µg/ml from a 50 mg/ml solution. Both were stored at -20°C.

2.13.2. Selection of positive colonies

Individual colonies (white colonies where blue/white selection was employed) were picked using a sterile pipette tip, and added to 150 µl of L-broth, containing an appropriate concentration and type of selective antibiotic, in a microtitre plate, then into the equivalent well of a PCR plate, containing PCR mix, with appropriate primers (see section 2.17.4). The culture plate was incubated in a shaking incubator, 200 rpm, for 1-3 h at 37ºC. Colonies positives by PCR were inoculated from the 96 well culture plate to grow minipreps, then further analysed by restriction digestion.

2.13.3. Storage of bacterial culture

Glycerol stocks were generated for each plasmid built. 700 µl of overnight culture and 300 µl of 50% glycerol were added to a labelled 1.8 ml Nunc tube (Thermo Scientific, Roskilde, Denmark). The tube was vortexed to mix and glycerol stocks were stored at -80ºC. To reanimate, the frozen stock was scraped with a sterile pipette tip, and streaked on an agar plate containing the appropriate antibiotic. Two colonies were picked, from this plate, added to 3 ml of LB agar containing the appropriate selective antibiotic and grown overnight, in a shaking incubator 250 rpm at 37ºC for miniprep purification.

2.14. Oligonucleotide synthesis

Custom oligonucleotides were synthesised by Invitrogen on a 25 nM scale. Oligos were received as a lyophilised pellet and were re-suspended in TE buffer [10 mM Tris, pH8.0, 0.1 mM EDT] to generate a 100 µM stock solution. Stock solutions were stored at -20ºC and were used for further dilutions in TE buffer. Oligos used in this study are listed in appendix 1.

2.15. Polymerase chain reaction

2.15.1. Standard PCR

Where fidelity of amplified DNA was not critical, DreamTaq™ DNA polymerase (Fermentas) was used for amplification of DNA. The reaction mix was set up according to manufacturer's the instructions: 5 µl of 10× DreamTaq™ polymerase buffer, 1 µl dNTP Mix (10 mM) (Roche), 2 µl of each primer (10 µM), 0.5 – 1 µg of

DNA template, 0.7 µl of 5 U/µl of DreamTaq™ polymerase, to a final volume of 50 µl. Cycling procedures were typically the following:

94ºC for 1 min to ensure denaturation of template

5 cycles of:

Denature at 94ºC, 10 s

Anneal at 58ºC, 45 s

Extend at 68ºC, 1 min per kb of DNA

Followed by 5 cycles of:

Denature at 94ºC, 10 s

Anneal at 55ºC, 45 s

Extend at 68ºC, 1 min per kb of DNA

Followed by 30 cycles of:

Denature at 94ºC, 10 s

Anneal at 52ºC, 45 s

Extend at 68ºC, 1 min per kb of DNA

Then a final 6 min extension step at 68ºC.

For all PCRs, annealing temperatures were primer-dependent.

2.15.2. High fidelity PCR

When error free cloning was required, a proof-reading DNA polymerase was used for PCR. The polymerases are all thermostable and exhibit a proofreading 3' to 5' exonuclease activity. The polymerases of choice were *Pfx50*™, Platinum® *Taq* High Fidelity Polymerase, or Accuprime™ *Pfx* High Fidelity Polymerase (Invitrogen).

Annealing temperatures depended on the primers used, and the extension temperature depended on the proof-reading enzyme used.

2.15.2.1. AccuPrime™ *Pfx* DNA Polymerase PCR

The AccuPrime™ *Pfx* DNA polymerase (Invitrogen) reaction mix was set up as described in the manufacturer's protocol, as follows: 2.5 µl of 10× AccuPrime ™ *Pfx* reaction mix, 0.3 µl of each primer (10mM each), up to 200 ng of DNA template, 0.5 µl of AccuPrime™ *Pfx* DNA polymerase, final volume of 25 µl with water. Cycling procedures were typically:

95ºC for 2 min to ensure denaturation of template

35 cycles of:

Denature at 95ºC for 15 s

Anneal at 55-64ºC for 30 s

Extend at 68ºC for 1 min per kb of DNA

Then a final 7 min extension step at 68ºC

2.15.2.2. *Pfx50*™ DNA Polymerase

The *Pfx50*™ DNA Polymerase (Invitrogen) reaction mix was set up according to manufacturer's directions, as follows: 5 µl of 10× *Pfx50*™ PCR mix, 1.5 µl dNTP mix (10 mM), 0.5 µl of each primer (10 µM each), up to 100 ng or up to 200 ng of plasmid or genomic DNA template, respectively, 1 µl of *Pfx50*™ DNA Polymerase, final volume of 50 µl with water. Cycling procedures were typically as follows:

94ºC for 2 min to ensure denaturation of template

35 cycles of:

Denature at 94ºC for 15 s

Anneal at 60-68ºC for 30 s

Extend at 68ºC for 1 min per kb of DNA

Then a final 5 min extension step at 68ºC

2.15.2.3. Platinum® *Taq* DNA Polymerase High Fidelity

The Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) reaction mix was set up according to manufacturer´s directions as follow: 5 µl of 10× High Fidelity PCR buffer, 1 µl of dNTP mix (10 mM), 2 µl of $MgSO_4$, 0.5 µl of each primer (10 µM each), 2 µl of DNA template, 0.2 µl of Platinum® *Taq* High Fidelity, final volume of 50 µl with water. Cycling procedures were typically:

94ºC for 2 min to ensure denaturation of template

35 cycles of:

Denature at 94ºC for 30 s

Anneal at 55ºC for 30 s

Extend at 68ºC for 1 min per kb of DNA

Then a final 5 min extension step at 68ºC .

2.15.3. Reverse transcription (RT) PCR

SuperScript® III First-Strand Synthesis System (Invitrogen) was used to synthesise first-strand cDNA from total RNA. After extraction and quantification of total RNA (sec. 2.6.3.), 1 µg total RNA was treated with DNase I (Roche) to remove any contaminating genomic DNA from the RNA sample: 1 µl of RiboLock™ RNAse Inhibitor (Fermentas), 1.5 µl of 10× DNase I buffer, 1 µl/2 U DNase I, and RNAse-free water up to 15 µl were added to the RNA. After incubation at 37ºC for 15 - 30

min, the DNase I was inactivated by adding 1 µl of 25 mM EDTA to the reaction mix and incubating it for 10 min at 65ºC. cDNA first strand synthesis was performed by adding to the DNase I-treated RNA, 2.5 µl of OligodT (10 µM), 2.5 µl of dNTP mix (10µM each), and 12 µl of RNAse-free water and incubated in a PCR block at 65ºC for 5 min. After incubation, 10 µl of 5× First strand buffer, 5 µl 0.1 M DTT and 1 µl/40 U of RiboLock™ RNAse Inhibitor (Fermentas) was added to the reaction mix and it was incubated for 2 min at 25ºC. 1 µl/200 units of Superscript™ Reverse Transcriptase was then added to start the reaction. At the same time two 'no-RT' controls were set up, consisting of all the reagents above, except for the reverse transcriptase. Reactions were incubated in a thermocycler for 10 min at 25ºC, 50 min at 42ºC and 15 min at 70ºC. cDNA was stored at -20ºC.

RT-PCR was performed with cDNA template in order to establish the presence or absence of a transcript within a tissue or life stage. Primers spanning intron/exon boundaries were used, as a further control against genomic contamination. PCR was set up as follow: 2.5 µl of 10× DreamTaq™ Polymerase buffer, 5 µl Betain solution (Sigma-Aldrich, Gillingham, UK), 0.5 µl dNTP mix (10 mM), 0.75 µl of each primers (10 µM), 0.5 ng to 1 µg of cDNA, 0.3 µl of 5 U/µl of DreamTaq™ polymerase, to a final volume of 25 µl. Cycling procedures were typically:

95ºC for 1 min to ensure denaturation of template

30 cycles of:

Denature at 94ºC, 15 s

Anneal at 50ºC, 30 s

Extend at 72ºC, 1 min per kb of DNA

Followed by 5 cycles of:

Denature at 94ºC, 15 s

Anneal at 50ºC, 30 s

Extend at 72ºC, 1 min per kb of DNA

Followed by 5 cycles of:

Denature at 94ºC, 15 s

Anneal at 50ºC, 30 s

Extend at 72ºC, 1 min per kb of DNA

Pause at 4ºC

Annealing temperatures were primer-dependent.

2.15.4. Colony PCR

A colony was picked using a sterile pipette tip and mixed into 10 µl of PCR mix made up of: 1 µl of 10× DreamTaq™ polymerase buffer, 0.2 µl dNTP mix (10 mM), 0.2 µl of each primers (10 µM), 0.07 µl of 5 U/µl of DreamTaq™ polymerase and 8.33 µl of water. Cycling procedures were typically as follows:

94ºC for 1 min to ensure denaturation of template

3 cycles of:

Denature at 94ºC, 15 s

Anneal at 60ºC, 45 s

Extend at 68ºC, 1 min per kb of DNA

Followed by 3 cycles of:

Denature at 94ºC, 15 s

Anneal at 57ºC, 45 s

Extend at 68ºC, 1 min per kb of DNA

Followed by 24 cycles of:

Denature at 94ºC, 15 s

Anneal at 54ºC, 45 s

Extend at 68ºC, 1 min per kb of DNA

Then a final 6 min extension step at 68ºC.

For all PCRs, annealing temperatures were primer-dependent.

2.16. Adding restriction sites by PCR

Where the restriction sites within a plasmid's multiple cloning site were not suitable for the insert, restriction sites compatible to the insert were generated by PCR. Two sets of primers were designed with one or more new restriction sites. Oligonucleotide primers used in the PCR were designed with restriction sites added to their 5' regions. Amplification generated a fragment whose termini carried new restriction sites compatible with the vector's restriction sites. A tail of six nucleotides was added to the 5' end as some restriction enzymes are inefficient at cleaving close to DNA ends. This allows the restriction enzyme to bind and cleave the restriction site more efficiently. The purified insert and vector were digested with the appropriate enzymes and ligated together.

2.17. Generation of oligonucleotide adaptors

Where a vector required the addition of extra restriction enzyme sites or insertion of a small DNA fragment adaptors were used. Two sets of complementary oligonucleotides were designed, containing desirable restriction sites and/or sequence of interest. These oligos overlapped except for 6 bp or 8 bp sequences, which were

compatible with the terminal restriction sites of the plasmids. Annealing was achieved by incubating equimolar amounts of each oligo (100 mM/ml), in 10× annealing buffer [100 mM Tris-HCl, pH7.5, 1 M NaCl, 10 mM EDTA], at 92ºC for 2 min, then a progressively reduced annealing temperature, 70ºC for 10 min, 65ºC for 5 min, 60ºC for 5 min, 55ºC for 5 min, 50ºC for 5 min, 45ºC for 5 min, 40ºC for 5 min, 35ºC for 5 min, 30ºC for 5 min and 25ºC for 5 min. Upon annealing, the primers formed a double-stranded DNA fragment with sticky ends (adaptor). The adaptor was then ligated into the plasmid.

2.18. DNA sequencing

DNA sequencing was performed by GATC Biotech (Konstanz, Germany). Constructs were sequenced using primers spaced 500-750 bp throughput the sequence to achieve 100% coverage. DNA fragments cloned into pJET1.2/blunt cloning vector (Fermentas) were sequenced with pJETFP2 and/or pJETRP2 primers (sequences in appendix 3).

2.19. Sequencing transgene genomic insertion sites

The genomic sequence flanking the inserted transgene was determined by using an adaptor-based amplification method that involves ligation of a double-stranded oligonucleotide adaptor to digested genomic DNA, followed by PCR with primers specific for the piggyback sequences and the adaptors.

Between 500-1000 ng of genomic DNA, from each transgenic line, was digested using the restriction enzymes *Bcl*I, *Bgl*I, *BSt*BI, *Cla*I, *Dpn*II, *HnP*1I, *Msp*I and *Nar*I

(New England BioLabs) (Figure 2.4 A). Double-stranded oligonucleotide adaptors, consisting of oligonucleotides '1338' and '1339' or '1350' (see appendix 3) were annealed by PCR as in section 2.17.1., and were ligated to the digested gDNA (Figure 2.4 B). Ligations were performed in a 20 µl reaction mix consisting of 10× T4 Ligase reaction buffer, 500-1000 ng of digested gDNA, 1 µl of adaptor (0.5 µg/µl), and 0.5 µl of 400 U/µL of T4 DNA Ligase (New England BioLabs), and incubated overnight at 16ºC. The adaptors have overhangs compatible with that produced by digestion with the above restriction enzymes (Figure 2.4 C). Thus, adaptors with a CG overhang will ligate to *Cla*I, *Bst*BI, *Msp*I and *Nar*I digested gDNA, and adaptors with a GATC overhang will ligate to *BclI*, *BglII* and *DpnII* digested gDNA. The genomic fragment adjacent to the *piggyBac* insertion site (between the adaptor and the construct's *piggyBac* internal sequence) was then amplified by PCR using an adaptor's specific primer (named 'Primer') and a primer specific for the internal *piggyBac* sequence at the 5' end (PB2) or the 3' end (PB3), at a ratio of 1:10 (Primer:PB) (Figure 2.4 D). PCR reaction mixes were made up as follows: 2.5 µl of 10× DreamTaq^{TM} buffer, 2 µl of PRIMER:PB mix (10µM), 0.5 µl of dNTP mix (10 mM), 0.5 µl of ligation reaction template, 0.2 µl DreamTaq polymerase and 17.3 µl water. Cycling was carried out as follow:

94ºC for 2 min to ensure denaturation of template

35 cycles of:

Denature at 94ºC, 20 s

Anneal at 55ºC, 45 s

Extend at 68ºC, 1 min 30 s

Then a final 9 min extension step at 68ºC.

A second nested PCR was performed using equal amounts of nested primers and 1:100 diluted first PCR product as template: 2.5 µl of 10× DreamTaq™ Buffer, 2 µl of MID primer (10µM, adaptor's specific), 2 µl of PB1 or PB3 primer (10µM), 0.5 µl of dNTP mix (10 mM), 1 µl of diluted first PCR product, 0.2 µl DreamTaq polymerase and 17 µl water. Cycling was carried out as follows:

94ºC for 2 min to ensure denaturation of template

35 cycles of:

Denature at 94ºC, 20 s

Anneal at 55ºC, 45 s

Extend at 68ºC, 1 min 30 s

Then a final 9 min extension step at 68ºC.

Amplified fragments form the nested PCR were gel-extracted, column-purified, cloned into pJET1.2/blunt cloning vectors (Fermentas), transformed, purified by Miniprep, and sent for sequencing with primers pJETFP2 and/or pJETRP2 primers. Sequencing data were analysed using VectorNTI (Invitrogen) and BLAST Analysis (National Centre for Biotechnology Information, NCBI, Bethesda, USA).

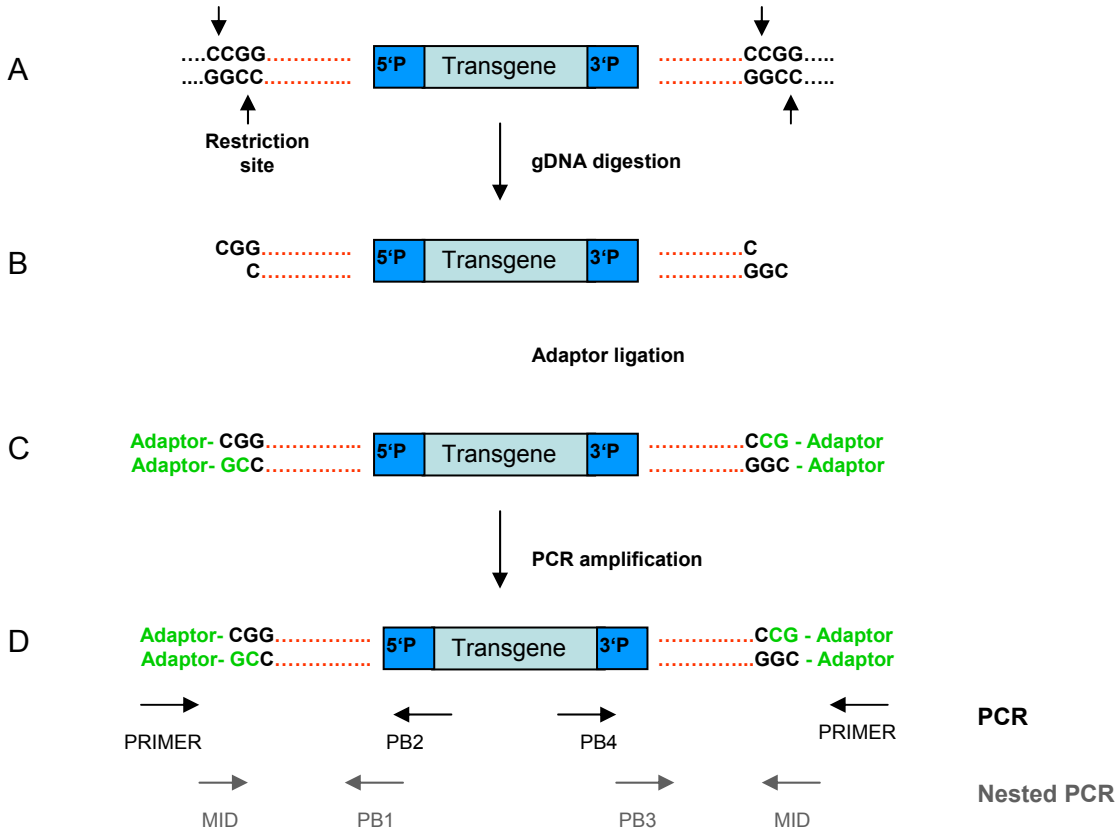

Figure 2.4. **Schematic representation of adaptor-based PCR amplification of unkown genomic sequences flanking and inserted transgene**. (A) and (B) Genomic DNA (**….**) is digested with a restriction enzyme that cuts approximately every 1-2 kb (↑), *Msp*I in the example. Note that the restriction enzymes might also cut inside the transgene. (C) Digested DNA is ligated to an adaptor (green) which has compatible overhangs with the restriction enzyme used to digest the gDNA. (D) Two rounds of PCRs are performed using primers specific to the adaptor and the *piggyBac* sequence (arrows) in order to amplify the genomic sequence adjacent to the *piggyBac* insertion site.

2.20. Rapid amplification of cDNA ends - 5' RACE

The amplification of the 5' end of a cDNA was performed using the SMART™ RACE cDNA Amplification Kit (Clontech). To amplify the 5' end of the cDNA, PCR was performed using an anti-sense oligonucleotide primer, called 'gene-specific primer' (GSP), that recognises a known sequence in the gene of interest and copies it in the 3'→5' direction, along with a primer specific to the kit adaptor (universal primer). A second nested PCR is then carried out, using a second nested anti-sense gene-specific primer (NGSP) that binds to the known sequence located upstream of

GSP within the cDNA product, along with a nested adaptor primer (nested universal primer A), resulting in specific amplification of cDNA ends.

First strand cDNA synthesis was carried out using 50 ng – 1 μg of total RNA, following the manufacturer's instructions. The 5'-RACE reaction was performed using Herculase II-Fusion DNA Polymerase (Stratagene, La Jolla, Canada), which provides successful amplification of complex and GC-rich templates. The reaction mix was set up as follows:

First-strand cDNA product	2.5 μl
GSP (1 μM)	1 μl
10× universal primer A Mix (UPM) (0.4 μM)	5 μl
dNTPs Mix (10 mM)	1 μl
5× HerculaseII-Fusion buffer	10 μl
HerculaseII-Fusion polymerase	0.75 μl
H_2O	Up to 50 μl

Cycling procedures were typically as follows:

5 cycles of:

Denature at 94ºC, 30 s

Extend at 72ºC, 30 s per kb of DNA

Followed by 5 cycles of:

Denature at 94ºC, 30 s

Anneal at 70ºC, 30 s

Extend at 72ºC, 30 s per kb of DNA

Followed by 25 cycles of:

Denature at 94ºC, 30 s

Anneal at 68ºC, 30 s

Extend at 72ºC, 30 s per kb of DNA

In order to perform the nested PCR, 5 µl of first PCR product was diluted in 245 µl of Tricine-EDTA buffer and the PCR reaction was set up as follows:

Diluted first PCR product	5 µl
NGSP (10 µM)	1 µl
Nested universal primer A (NUP) (10 µM)	1 µl
dNTPs mix (10 mM)	1 µl
5× HerculaseII-Fusion buffer	10 µl
HerculaseII-Fusion Polymerase	0.75 µl
H_2O	Up to 50 µl

Cycling procedures were typically:

20 cycles of:

Denature at 94ºC, 30 s

Anneal at 68ºC, 30 s

Extend at 72ºC, 30 s per kb of DNA

GSPs were designed according to manufacturer's directions: 23-28 nucleotides long, 50-70% GC and Tm ≥ 70ºC.

5' RACE PCR products were cloned into a pJET1.2/blunt cloning vector (Fermentas) and sent to sequence with primers pJETFP2 and/or pJETRP2 primers, at GATC. Sequencing results were analysed using VectorNTI (Invtrogen).

Chapter 3

Germline transformation of the diamondback moth

3.1. Introduction

The diamondback moth problem

The diamondback moth, *Plutella xylostella* (L.), is the most significant global pest of crucifers (vegetables in the family Brassicaceae, which include cabbage, broccoli, cauliflower and rapeseed). Considered the most widely distributed of all Lepidoptera, diamondback moth is present in all countries where host crops are cultivated. Control is primarily based on the use of chemical insecticides. Diamondback moth, however, is notorious for its capacity to rapidly develop resistance to chemical pesticides. Other, chemical-free means of control are also employed but conventional control methods have so far failed to adequately control the diamondback moth. Genetic manipulation of the diamondback moth could aid in the development of novel genetics-based control strategies, including the improvement of SIT programmes, providing another, much needed, control tool.

Genetic control of the diamondback moth

The sterile insect technique (SIT) (see Chapter 1, section 1.3) has been assessed as an environment-friendly, species-specific means of controlling diamondback moth (Okine et al., 1998; Omar, 1991; Sutrisno & Hoedaya, 1993; Sutrisno et al., 1991). SIT generally induces sterilisation by irradiation, which relies on costly and hazardous radiation sources, and may also have a detrimental effect on the performance of released insects (Robinson et al., 2004). Germline transgenic technology can overcome these problems associated with SIT: a method called Release of a Dominant Lethal (RIDL®) (see Chapter 1, section 1.5) has been shown to provide the effect of sterility (no viable progeny) in mosquitoes and fruit flies (Gong et al., 2005; Phuc et

al., 2007) and automated selection of males only (Fu et al., 2007; Fu et al., 2010). If diamondback moth could be transformed, RIDL may provide another chemical-free method of control.

Aim

This chapter aimed at establishing a germline transformation system for the diamondback moth. It was hoped that genetic manipulation for this species would open the way for both basic research and the development of new strains that could potentially be used in future control programmes.

3.2 Materials and methods

3.2.1. Generation of DNA constructs

3.2.1.1 OX4389

The OX4389 construct was made by modifying pB[3×P3-DsRed-nls-Sv403'UTR]*lox66* (Nimmo et al., 2006) to remove the 3×P3-DsRed marker and replace it with *Opie2*-nls-ZsGreen-nls marker. The 3×P3-DsRed cassette was removed using *Bst*BI/*Asc*I and the resulting vector was ligated to an *Asc*I/*Bst*BI adaptor, made of oligonucleotides '1000' and '1001' (see Apendix 3), and which contains internal restriction sites for *Nhe*I and *Swa*I. The resulting construct was then sequentially digested with *Asc*I/*Swa*I and ligated to an *Asc*I/*Swa*I *Opie2*-ZsGreen cassette obtained by digesting OX4343, pB[BmA3-tTAV-Opie2-DsRed-nls-SV40] with the same enzymes, *Asc*I/*Swa*I, creating OX4389 pB[*attP*-Opie2-nls-ZsGreen-nls-SV403'UTR].

3.2.1.2. Other constructs

Constructs used in this study which were made by colleagues:

OX1138**:** pB[*Opie2*-DsRed-nls-SV40], made by Dr Guoliang Fu

OX3441: pB[attP-Hr5*ie1*-DsRed-nls-SV40], made by Dr Guoliang Fu

OX4570: pB[3xP3-DsRed2-sv40], made by Dr Sarah Scaife

3.3. Results

3.3.1. Germ-line transformation

Three critical components of a transformation system were tested: transposable element (*piggyBac*) function, fluorescent protein and the regulatory sequence controlling its expression. Of the commonly used transposons, *piggyBac* has been used to transform the broadest range of insects, including the Lepidoptera *B. mori* (Tamura et al., 2000), pink bollworm (Peloquin et al., 2000) and the squinting bush butterfly (*Bicyclus anynana*) (Marcus et al., 2004). Therefore, *piggyBac* was selected to attempt germ-line transformation. The red fluorescent proteins DsRed2 and the green fluorescent protein ZsGreen (Clontech Laboratories Inc, Mountain View, CA) were selected as selectable markers. ZsGreen, is a fast-maturing green fluorescent protein, that has regulated strong marker expression in transgenic *Drosophila melanogaster* (Sarkar et al., 2006). It has also been shown to consistently give brighter fluorescence than other green fluorescent proteins - EGFP and TurboGFP - in *L. cuprina* (Concha et al., 2007). The red fluorescent protein DsRed2 is an improved variant of thee original DsRed protein (Matz et al., 1999; Lukyanov et al., 2000). As DsRed has been successfully used in *D. melanogaster* (Sarkar et al., 2006) and the malaria mosquito, *Anopheles stephensi* (Nolan et al., 2002), it was predicted that the improved DsRed2 would perform as well or better than DsRed.

Three regulatory sequences were used to control expression of these marker proteins: Hr5*ie1*, *Opie2* and 3×P3. The *Opie2,* a promoter fragment from the *ie2* gene of baculovirus *Orgyia pseudotsugata* nuclear polyhydrosis virus, a pathogen of the Douglas-fir tussock moth (*O. pseudotsugata*). *Opie2* has been used to drive DsRed2 expression in pink bollworm (Simmons et al., 2007). The Hr5*ie1*, a fragment of the

immediate-early-1 (*ie*1) gene with the Hr5 enhancer, from the *Autographica californica* nuclear polyhydrosis virus (AcMNPV). Hr5*ie1* has been used as a transformation marker in *Anopheles gambiae* (Grossman et al., 2001), Mexican fruit fly (*Anastrepha ludens*) (Concha et al., 2007) and Mediterranean fruit fly (*Ceratitis capitata*) (Gong et al., 2005). In Lepidoptera it has been shown to function transiently (transient injection assays rather than in germline transformants) in embryos of the potato tuber moth, *Phthorimaea operculella* (Mohammed and Coats, 2004). The 3×P3 is an artificial eye-specific promoter constructed using three tandem repeats of the binding site (P3) of the photoreceptor-specific Pax-6/Eyeless transcriptional activator (Sheng et al., 1997). The 3×P3 has been used to transform *B. mori*, resulting in expression of EGFP in all life stages, including late embryos (Thomas et al., 2002). In other Lepidoptera, this promoter has been shown to function in *B. anynana* (Marcus et al., 2004) and in transiently in the cabbage moth, *Mamestra brassicae* (Mandrioli & Wimmer, 2003).

Four different transformation constructs were built, all carrying a different promoter-fluorescent protein-encoding genes combination and inverted terminal repeat sequences from the *piggyBac* transposable element (Figure 3.1). All constructs have single inverted terminal repeats, except OX3441, which is double-ended. Fluorescent protein gene, in all constructs, was flanked by a nuclear localisation signal. OX3441 and OX4389 carry an *attP* sequence for ΦC31-mediated site-specific recombination, work developed in Chapter 4.

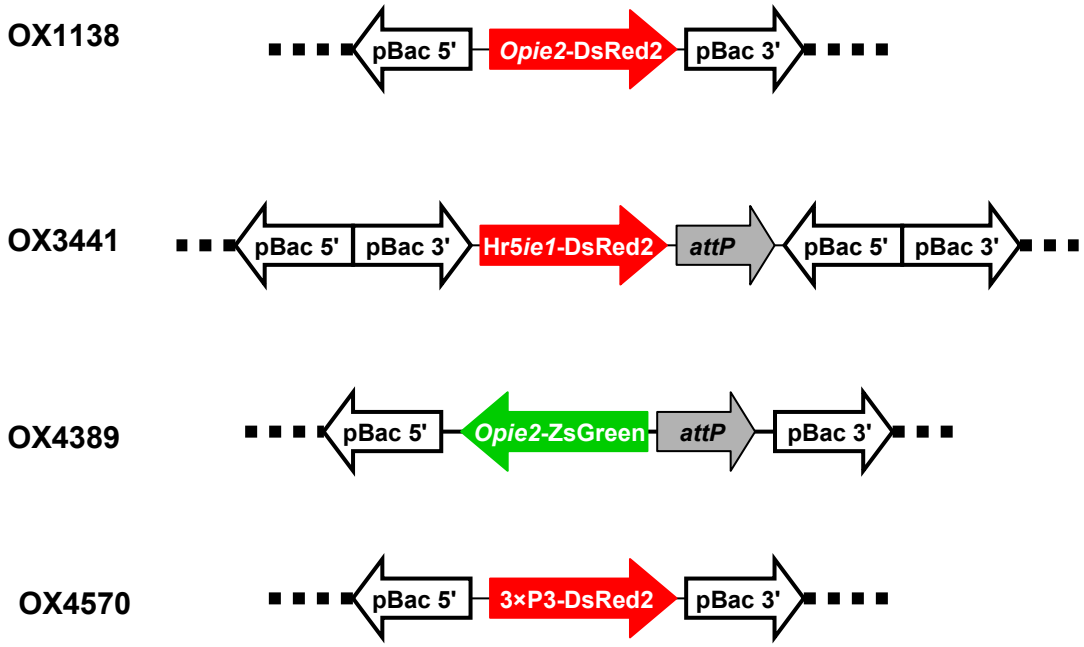

Figure 3.1. Schematic representation of constructs OX1138, OX3441, OX4389 and OX4570

All four constructs were co-injected with a non-autonomous helper plasmid, OX265 (Figure 3.2), originally used in the pink bollworm transformation by Peloquin et al. (2002), at 500 ng/µl and 300 ng/µl, respectively. In general, microinjection is conducted into the posterior of the embryos, opposite the micropyle (Steller & Pirrota, 1968) where the pole cells are located (Berger & Gassner, 1978). Location of the posterior end of the egg and/or of the pole cells has not been described in diamondback moth. Therefore, identification of the potential posterior end of the embryos was carried out by comparing diamondback moth embryos with pink bollworm embryos. The pink bollworm is, in our hands, a routinely transformed species and its eggs have a similar morphology to diamondback moth eggs. In the pink bollworm the posterior pole of the eggs is narrower and smoother than the anterior. Based on this, microinjections in this study were aimed at the narrower end of the embryos (see Chapter 2, section 2.2.3). After injection, transient expression of the fluorescent marker protein in surviving larvae and pupae - patches of fluorescence

in the abdomen - indicated that the plasmid had been injected in the approximate target location.

Figure 3.2. Schematic representation of helper plasmid OX265. The non-autonomous helper plasmid encodes the *piggyBac* (pB) transposase, under the control of the *Drosophila melanogaster hsp70* promoter fragment.

In total, between 1202 and 3104 diamondback moth wild-type embryos were co-injected with one of the above constructs and non-autonomous helper plasmid, source of transposase (Table 3.1). All injection survivors (G_0) that reached pupation were collected, separated by sex and out-crossed to wild-type mates at a male:female ratio of 1:3, in pools of 2-5 putative transgenics. G_1 progeny were screened for fluorescence as pupae. Multiple transgenic lines were obtained for each construct tested. Post-injection survival varied among constructs, ranging from 19% to 76% (Table 3.1). In comparison, Peloquin and colleagues achieved a 37% post-injection survival when they transformed pink bollworm (Peloquin et al., 2000) and Ferguson and colleagues achieved 16-47% survival with injections of codling moth (*Cydia pomonella*) (Ferguson et al., 2010). Much lower survival rates have been seen in *B. mori* - 14% by Thomas et al. (2002) and 11-26% by Uhlířová et al. (2002) - and *B. anynana* - 5% by Marcus et al. (2004). These differences between species might be best ascribed to the very diverse size and morphology of eggs among Lepidoptera, making some more easy to inject than others. User training and skill levels are also likely have a significant influence on injection survival rates. Transgenic G_1 animals

expressing the fluorescent phenotype were maintained as hemizygotes[1] at the insertion locus by backcrossing to wild-type mates.

Table 3.1. Microinjection data for four constructs in diamondback moth. Transformation efficiency is calculated as the proportion of injection survivors (G_0) that produce transgenic offspring (for broods containing more than one G_0, all transgenic G_1 were assumed to represent only a single independent event).

Construct	Marker cassette	Embryos injected	G_0 survivors to pupae (% survival)	No. of independent lines	Estimated transformation efficiency
OX1138[2]	*Opie2*-DsRed2	3104	584 (19%)	4	0.68%
OX3441	Hr5*ie1*-DsRed2	1925	1462 (76%)	›7	0.48%
OX4389	*Opie2*-ZsGreen	2410	922 (38%)	6	0.65%
OX4570	3×P3-DsRed2	1202	463 (39%)	3	0.65%

Transformation efficiency can be defined as the proportion of fertile G_0 injection survivors producing at least one transformed G_1 progeny. Due to the high number of G_0 survivors, G_0 survivors were pooled to reduce labour. It is therefore not possible to determine the sterility/fertility rate of the injection survivors or to calculate the precise transformation efficiency. The estimated transformation efficiency ranged between 0.48% and 0.68% (number of independent lines as proportion of total G_0 survivors).

This is a minimum estimate of the transformation efficiency as it has been assumed that in each cross, composed of more than one G_0 individual, all transgenic G_1 represent a single transformation event. This estimate also assumes that all individuals

[1] Transgenic insects carrying a dominant allele (transgene) are troughout this study, termed hemizygous as they carry only one copy of a genomic region that has no allelic counterpart. Therfore, by definition, they are not heterozygous because the transgenic allele is missing from the homologous chromosome.

[2] Injections of the OX1138 construct were performed by Neil Naish

in each cross were fertile and mated to give screenable progeny which, in reality, is not likely to be the case. For comparison, Peloquin et al. (2000) achieved a transformation efficiency of 3.5% when transforming the pink bollworm. Tamura et al. (2000) achieved 0.7% and 3.9% when transforming the silkworm.

3.3.2. Fluorescent protein expression

Individuals were easily detected and distinguishable from non-fluorescent individuals. The *Opie2* sequence drove strong expression of ZsGreen and DsRed2 (OX4389 and OX1138 lines, respectively) widely and evenly throughout visible tissues of the pupae (Figure 3.3). In both cases, close examination showed that this expression was composed of foci of fluorescence, presumably a result of the nuclear localisation signal sequences adjacent to the fluorescent protein coding sequences on the transgene. This can be an important tool to distinguish between true fluorescence and background fluorescence emitted by insect tissues and gut content. The Hr5*ie1* enhancer-promoter also drove high levels of DsRed2 expression in OX3441 lines. In comparison, DsRed2 expression in OX1138 lines (*Opie2*) was less strong and more uneven in distribution, both in larvae and pupae. All transgenic lines appeared to develop at a rate similar to that of their wild-type siblings. Transgenic individuals carrying the *Opie2*-marker and Hr5*ie1*-marker constructs showed no visible differences in fluorescent protein expression between lines carrying the same construct. One of the strains carrying the 3×P3 marker cassette showed stronger expression of DsRed2 than the other two strains.

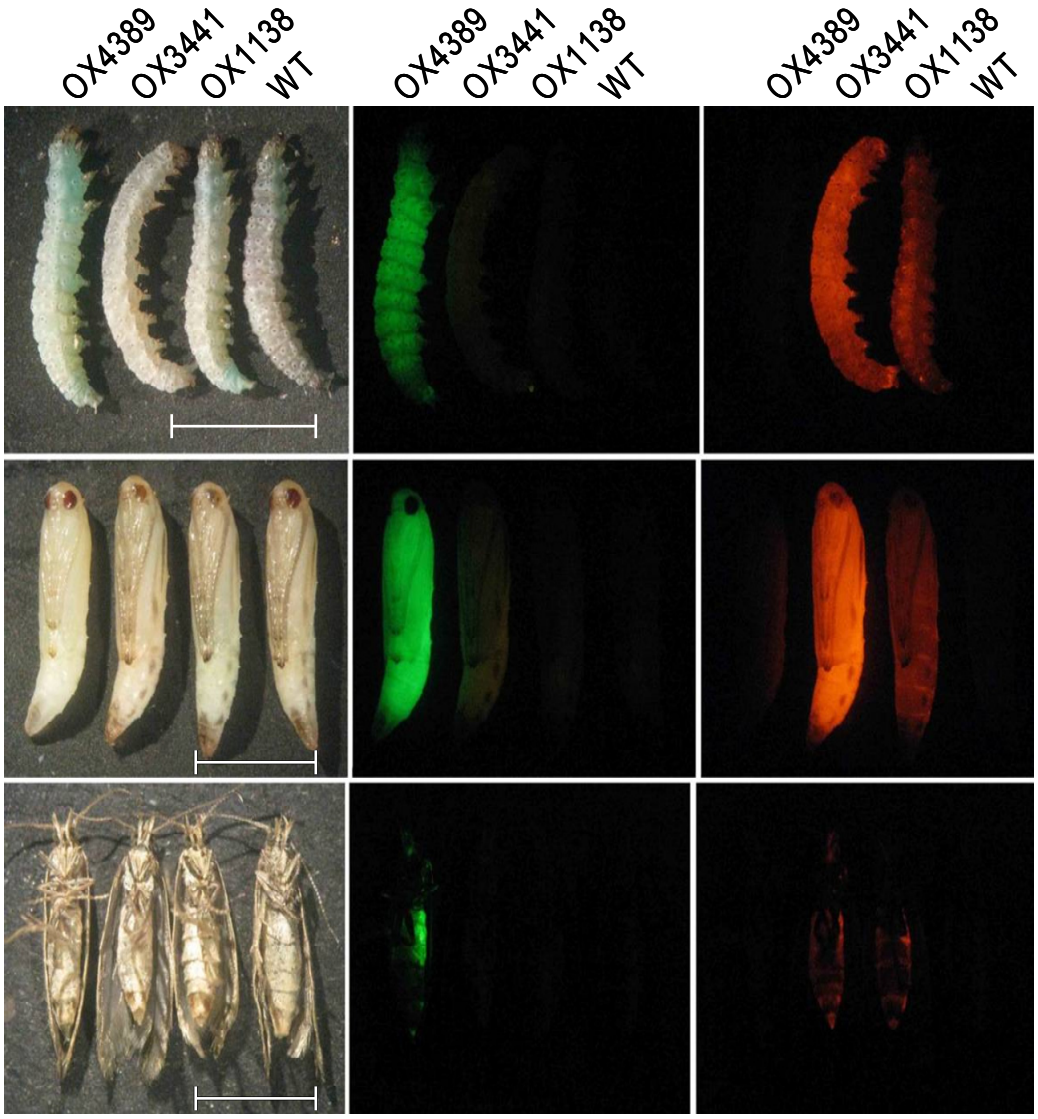

Figure 3.3. Photographs showing fluorescent protein expression in transgenic and wild-type diamondback moth at different life stages; larvae (top panels, bar scale size=5 mm), pupae (middle panels, scale bar represents 3.5 mm) and adults (lower panels, scale bar represents 3.5 mm). The left-hand panels show the insects in white light, the middle panels under green fluorescence excitation light and filters, and the right-hand panels under red fluorescence excitation light and filters. In all panels, the insects are arranged as follows (left to right): transformed with OX4389, OX3441, OX1138 and wild-type.

In OX4570-transformed lines, DsRed2 fluorescence driven by the 3×P3 promoter was clearly visible in the eyes of G_1 individuals (Figure 3.4). As the pupa matures, the eyes become darker, heavily pigmented with ommochromes, making the detection of the fluorescent protein more difficult in OX4570 lines. These expression patterns are similar to those regulated by 3×P3 in transgenic *B. mori* (Thomas et al., 2002) and *B.*

anyana (Marcus et al., 2004). Fluorescence intensity was uniform between individuals of the same line, but not between lines. DsRed2 expression in line OX4570A was less strong than in OX4570B and OX4570C. These differences are likely an effect of position effect (Bell & Felsenfeld, 1999; Wilson et al., 1990) or a transgene copy number effect. The influence of the genomic sequence surrounding the transgene insertion has also been reported in other transgenic insects (Allen et al., 2004; Fu et al., 2010; Handler & Harrell, 1999, 2001b; Horn et al., 2002; Labbé et al., 2010).

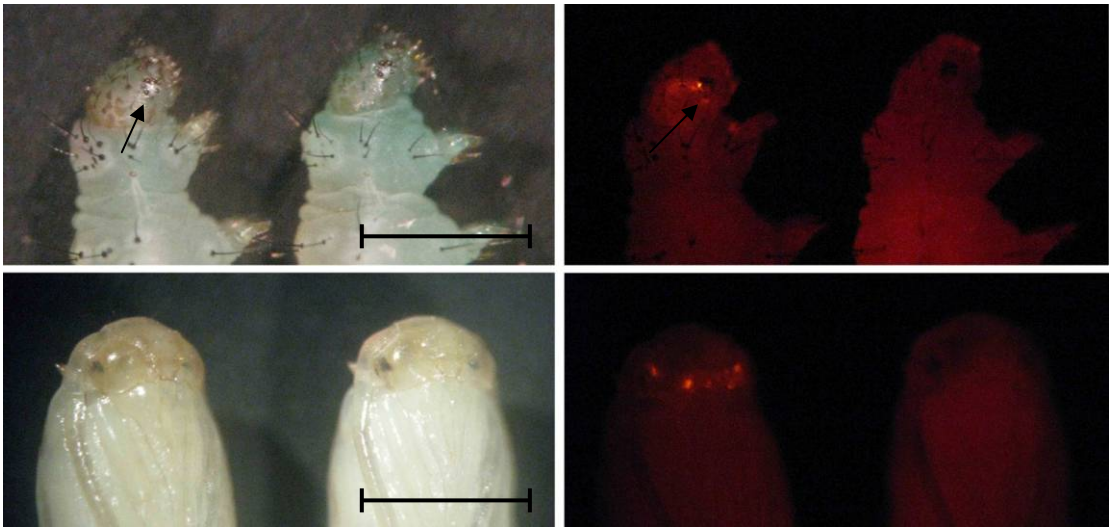

Figure 3.4. Photographs showing 3×P3-regulated DsRed2 expression in OX4570B-transformed larvae and pupae. The left-hand panels show the insects in white light and the right-hand panels under red fluorescence excitation light and filters. In all panels, the OX4570B insects are on the left, and the wild-type on the right. Scale bar represents 1 mm. Arrows indicate the stemmata.

All regulatory sequences induced fluorescent protein expression in larvae (all instars), pupae and adults. In addition, the Hr5*ie1*-DsRed2 (OX3441), *Opie2*-DsRed2 (OX1138) and *Opie2*-ZsGreen (OX4389) marker cassettes showed visible fluorescence in late embryos, after developing larval heads became visible.

In OX4570 strains, the 3×P3 promoter drove strong eye-specific expression of DsRed2 throughout all post-embryonic stages of development. 3×P3 was active in the peripheral and central nervous system of the larvae, similar to that described in *B. mori* (Dai et al., 2007; Thomas et al., 2002). Dai and colleagues detected GFP expression in the stemmata (larval eyes) and nervous system of *B. mori* embryos aged 4 days and older. This was not detected in mature diamondback moth eggs. Low levels of DsRed2 may have been locally expressed, but the relatively small size of the eggs would have made this difficult to see without dissection.

3.3.3. Molecular confirmation of *piggyBac*-mediated insertion

In order to verify that the genomic insertion of the transgenes, the genomic sequences at the insertion sites flanking the integrated construct were determined. All of the sequences determined included the TTAA duplication at the construct insertion sites (Table 3.2) characteristic of *piggyBac*. At least one side of the genomic insertion was sequenced for each line, apart from OX3441F. Repeated efforts to determine this sequence were unsuccessful. Full flanking sequencences can be found in appendix 4.

Table 3.2. Genomic DNA sequences flanking transgene insertions of independent transgenic strains of diamondback moth. The TTAA duplicated sequence is present at all 5′ and 3′ insertion boundaries (ND – sequence not determined). Efforts to sequence either side of the insertion site of OX3441F were unsuccessful.

Construct	Line	5′-end genomic flanking sequence		3′-end genomic flanking sequence
OX4389	A	CGGCTGGGGCAGATATTTGT	TTAA	ACACAGATATTTGTTCTCGG
	B	GACTAACCTTCGAGTCGTGT	TTAA	GCAACAAATCTGTTCTGCTA
	C	CAAAACTTCAGACAGCTTTT	TTAA	AAATGGAATACCATTGCGAT
	F	TATCGCTAAATATTTTCTTT	TTAA	AAATCTTTTTTTGTAATATT
OX4570	A	GAAGTAATTATTTCGTTTGA	TTAA	ACGTAAAATATGGTGTGATC
	B	GTCAAGCAACAGCAACTGGC	TTAA	ND
	C	TATCACTACGCACAGACCCA	TTAA	GGAAGTAATATATAAGTTGG
OX3441	A	TGCATTGTATGTTGTATTCG	TTAA	ND
	B	AAAGTTAAGTTATCACTTTA	TTAA	ND
	C	CCCAATGGTATAAATTGTAC	TTAA	ATACCAAAGAACTCATTGGTA
	D	ND	TTAA	GTGAGTAGATAATTGTGTCTT
	F	ND	TTAA	ND
	G	TGGTTCTCTGAAGTACGTAT	TTAA	ND
	H	ACGTAAACTCATTTTCGTCT	TTAA	AACTCTACGATTTGTTGCCTC
OX1338	A	AAAATTACAAAACTTCTTGT	TTAA	CAAAGTGTTTACTTAAGTGG
	B	CGTATATCGTTTTATTTTCC	TTAA	ND
	C	AAAATGGGACAGCCGCGGTG	TTAA	ND
	D	CCACTTAAGTAAACACTTTG	TTAA	ND

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) nucleotide analysis was carried out on these genomic flanking sequences. For all of the lines, except for 4389A and 3441C, no similarities were found, which might be expected as the diamondback moth genome has not been fully sequenced. The 5′- and 3′-end flanking genomic sequences of OX4389A and the 5′ flanking sequence of OX3441C showed similarity to diamondback moth microsatellite Pxy 038, 100 and 044 and diamondback moth microsatellite pxy065 sequence, respectively.

OX3441 carries four *piggyBac* transposable ends, which provided the opportunity to perform a test for potential remobilisation of the transposon in diamondback moth. The idea of using two adjacent pairs of *piggyBac* transposable sequence at each end of the construct was developed by Dafa´alla et al. (2006). The aim was to be able to re-mobilise the double ends after integration by either injecting the insect with a transposase source or by crossing it to a ´jumpstarter´ transgenic strain containing a transposase expression cassette. Once the double *piggyBac* ends were re-mobilised, the strain would be rendered *piggyBac*-free, ensuring transgene stability. Transgene stability is a potential requisite when releasing transgenic insects into the wild due to the remote possibility of horizontal transfer to untargeted wild species. Some flanking sequences were not determined for some OX3441 lines despite several attempts. This process proved to be challenging: using PCR-based methods to attempt amplification of the insertion flanking sequences, the presence of multiple pairs of *piggyBac* ends - a binding site for most primer pairs for these PCRs (see Chapter 2, Figure 2.3) – caused the internal sequences of the construct to be amplified and sequenced. These internal sequences were preferentially recovered relative to amplification from construct to genomic flanking sequence. It is possible that this preference for the internal construct-only amplification was due to reaction bias towards smaller fragments. True genomic flanking PCR amplifications generally produced larger fragments, although this was not always the case. In order to determine if failure to determine the flanking sequence could be due to loss of one or both *piggyBac* pairs or difficulties experienced with the flanking method, PCRs on genomic DNA of each OX3441 lines was performed (Figure 3.5) using a primer designed over the *piggyBac*-vector junction, to show the presence of the *piggyBac* element, and an internal primer.

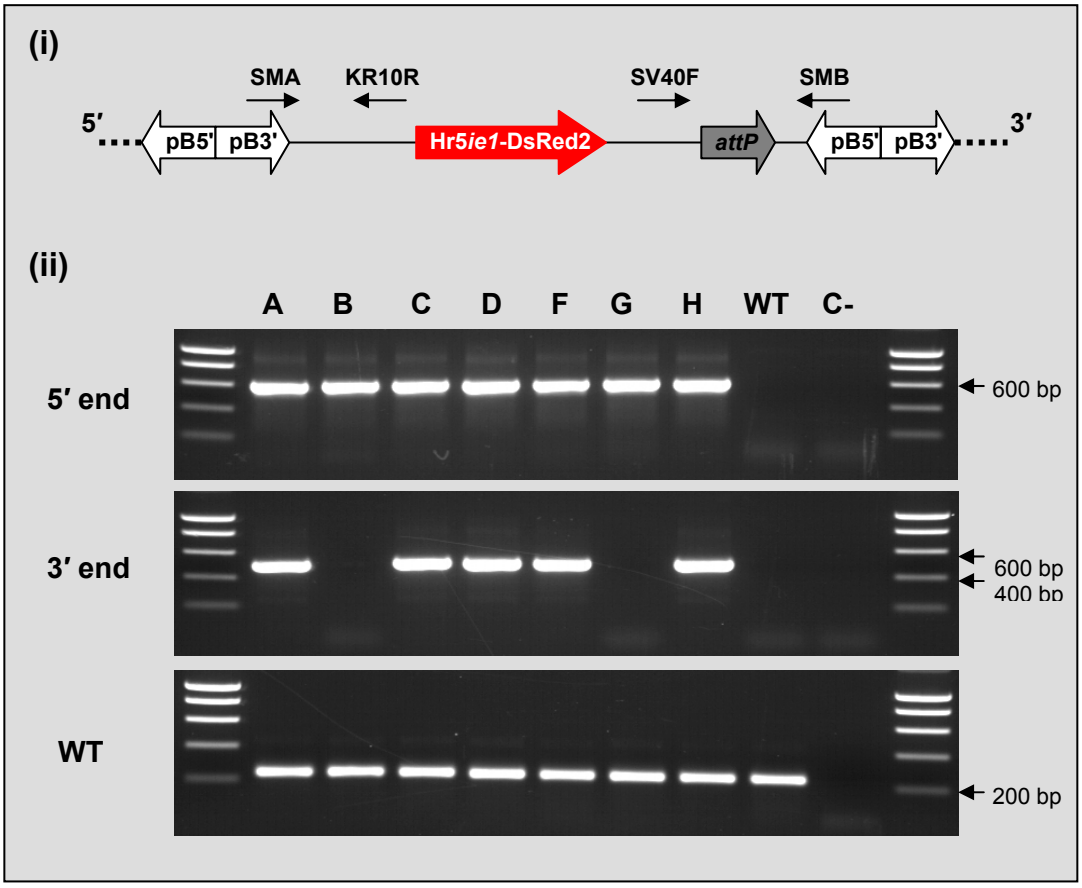

Figure 3.5. PCR on the OX3441 lines. (i) Schematic representation of construct OX3441 and primer SMA, KR10R, SV40F and SMB binding sites (primer sequences can be found in appendix 3). (ii) PCR amplifications; the 5′- and 3′- ends of the construct were amplified using primer pairs K10R/SMA (604 bp) and SV40F/SMB (487 bp), respectively. A wild-type genomic DNA (WT) and a no-DNA control (C-) were included in each PCR. A wild-type control PCR, using primers 4319Nf2 and 4319Nr1 (274bp) was also included. Smart ladder is run at both ends.

All lines appear to carry the *piggyBac* pair at the 5′ end of the construct, but only lines A, C, D, F and H carry the *piggyBac* pair at the 3′ end of the construct. It seems that lines B and G have lost the *piggyBac* pair at the 3′ end of the construct. It is possible that which such an OX3441 construct design, excision of the entire construct could is also possible. In such case, trangenic individuals would return to a non-fluorescent, 'wild-type' state and would be impossible to identify in a hemizygous strain as the one used in this study. Furthermore, since shorter transposons may transpose

morereadily than longer ones (Dafa'alla et al., 2006), excision of *piggyBac* pairs is more likely to occur then excision of the entire construct.

Mendelian inheritance of the fluorescent markers over several generations for at least eight generations for most lines generated indicates that the *piggyBac* vectors were stably maintained in the insects' genome (Table 3.3). Chi square analysis (χ^2) showed that the ratio of the number of transgenics and wild-type produced by each line is not significately different from 0.5. This is consistent with each line carrying a single insertion. It was not possible to generate Mendelian data for OX1338B, C and D lines as these lines were lost.

Table 3.3. Mendelian inheritance of the transgene in OX4389, OX4570, OX3441 and OX1138A lines. The significance of the difference in observed transgenic:wild-type ratio and the expected 50:50 ratio, predicted by Mendelian genetics, was calculated by Chi square analysis (χ^2), where $p<0.05$ represents a significant difference. NA=not available.

		Line	Transgenic	Wild-type	χ^2 (df=1)	p value
Construct	**OX4389**	A	119	144	2.376	0.1232
		B	99	122	2.394	0.1218
		C	183	174	0.227	0.6338
		F	176	183	0.136	0.7118
	OX4570	A	200	208	0.157	0.6921
		B	197	192	0.064	0.7999
		C	202	195	0.123	0.7253
	OX3441	A	104	108	0.075	0.7835
		B	68	69	0.007	0.9319
		C	92	96	0.085	0.7705
		D	140	135	0.091	0.7630
		F	97	97	0.000	1.0000
		G	114	134	1.613	0.2041
		H	96	72	3.429	0.0641
	OX1138	**A**	135	141	0.130	0.7180
		B	NA	NA		-
		C	NA	NA		-
		D	NA	NA		-

3.4. Discussion

This chapter reports the use of a *piggyBac*-based system to achieve the first successful germline transformation of the diamondback moth, extending to five the number of lepidopteran species that have been transformed with this transposon. It represents an important step towards the establishment of routine transformation methods in this major pest species.

Results obtained show that a *piggyBac*-derived vector is able insert into the diamondback moth genome in a stable manner. The two selectable marker proteins tested - DsRed2 and ZsGreen - generally fluoresced brightly and were easily screened. It is important that true fluorescence is distinguishable from background autofluorescence so that transgenic individuals can be easily separated from non-transgenic siblings. This has been a problem in previous transformation systems in other insects (Handler & Harrell, 2001a; Heinrich et al., 2002; Horn et al., 2002). The use of a nuclear localisation signal with the fluorescent proteins might also aid the identification of transgenic individuals especially when marker expression is weaker. When a fluorescent protein is nuclear-localised, the fluorescent protein accumulates in the cell nucleus producing foci of fluorescence. This gives a distinct spotty appearance to transgenic individuals. Nevertheless, expression from these promoters was strong enough to clearly separate transgenic from non-transgenic individuals. Finally, three different regulatory sequences were shown to drive fluorescent protein expression in diamondback moth. Both *Opie2* and Hr5*ie1*, isolated from lepidopteran nuclear polyhedrosis viruses were expected to be transcribed by host factors upon

insertion. As expected, both promoters drove the expression of both fluorescent markers in many tissues, through most life stages (not early embryos). We also demonstrated function of the 3×P3 promoter in diamondback moth, although marker expression driven by this promoter is not as easily detected at late pupal stages as that regulated by *Opie2* and Hr5*ie1*.

Transformation rate that we have achieved in diamondback moth (0.48-0.68%) is lower than that described with *piggyBac* in silkworm (0.7-3.9%) (Tamura et al., 2000) and pink bollworm (3.5%) (Peloquin et al., 2000). The lower recorded diamondback moth transformation efficiency may be due to inherently lower activity of *piggyBac* in diamondback moth. In addition, the eggs of diamondback moth are smaller and therefore less easily injected. However, this lower efficiency is not prohibitive to regular transformation of diamondback moth and as, pooled injection survivors crosses were made instead of single crosses, it is not possible to determine the exact transformation efficiency. Thus, transformation rates obtained here should be viewed as minimum estimates.

Flanking and PCR analysis of the double-ended *piggyBac* OX 3441 lines suggest that *piggyBac* remobilisation might happen in the diamondback moth (Table 3.2 and Figure 3.5). This is particularly interesting as one objection to the release of transgenic insects has been the theoretical possibility of transposase-mediated instability of the transgene in the insect genome, in the unlikely event of the transgene being exposed to exogenous transposase (Condon et al., 2007). Transposon-free insertions, resulting from post-integration remobilisation of *piggyBac* ends, would eliminate this concern. However, further confirmations that strains can be made *piggyBac*-free and stable is needed, for example by crossing the OX3441 lines to a "jumpstarter" line (source of transposase) or by injecting transposase helper plasmid

into the embryos of these lines to test if remobilisation of both ends is possible. This has been proven to be possible in fruit flies (Condon et al., 2007), *Anopheles stephensi* (O´Brochta et al., 2011) and in the pink bollworm, but not in *Ae. aegypti* (Neil Morrison, personal communication).

These results provide encouragement for future efforts to develop new methods of control for this globally important pest such as the development of RIDL technology in diamondback moth to overcome obstacles that have so far prevented SIT from being implemented against this pest. In addition, with its short life cycle and easy rearing in the laboratory, transgenic technology in diamondback moth opens the door to gene function studies and much wider research using this moth as a model species.

Chapter 4

Site-specific engineering of diamondback moth

4.1. Introduction

Current methods for insect transformation rely on random[3] integration of exogenous DNA into the insect´s genome using transposon-based vectors. Random insertion of a transgene into the genome introduces variation in transgene expression due to position effects. These result from the influence on the transgene of local regulatory elements, such as nearby enhancers or repressors, and/or the architecture of the surrounding chromatin, which may modify the expression of the transgene (Wilson et al., 1990; Wimmer, 2005; Bhalla, 1968; Jasinskiene et al., 1998; Coates et al., 1998). Therefore, transgene expression levels and patterns can differ between independent lines carrying the same construct (Wilson et al., 1990), indeed this phenomenon has been exploited in 'enhancer trap' screens. Introducing uncertainty in the engineering of phenotypes means that multiple insertions may need to be screened for target phenotypes, or to determine the effect of a construct element separate from confounding effects of the insertion site(s). In addition, the molecular characterisation of neighbouring genomic sequence is necessary for individual lines to be able to strictly distinguish between strains and to PCR-genotype when generating transgene-homozygous strains, which can be time-consuming. On the other hand, position effect allows a degree of fine-tuning of phenotype without adjusting the construct.

Site-specific recombination

One way of mitigating the effects of insertion site is to insert all transgenes (or the set of interest) into a single genomic location, where they would all be subject to the

[3] Strictly, pseudo-random. *piggyBac*, for example, has a short target sequence TTAA so will not insert literally anywhere, not at any of an infinite number of sites. However, this target sequence occurs a very large number of times per genome, so insertions are effectively random, on a genomic scale.

same position effect, so that variations in phenotype should be directly attributable to differences in construct sequence. This can be achieved by site-specific recombination.

Site-specific recombination systems are well known in bacteria and lower eukaryotes (Lyznik et al., 2007) and are involved in several biological processes such as insertion of phage DNA into the host chromosome and the maintenance of the structural integrity and copy number of circular DNA molecules during cycles of DNA replication and cell division (Sherratt, 2001; Saraf-Levyet al., 2006; Grindley et al., 2006).

Site-specific recombination systems are based on the specific interaction of a recombinase with its cognate DNA target sequence(s), termed recombination sites. The recombinase recognises and binds to two recombination sites, forming a synaptic complex, and then catalyses cleavage, strand exchange, and rejoining of the two cleaved DNA segments (Grindley et al., 2006). The recombination sites may be identical or different, and unlike general recombination processes, strand exchange requires relatively little sequence homology between the two recombination sites (Smith et al., 2004). Depending on the initial arrangement (*cis* or *trans*) and orientation of the target sites, the outcome of a recombination reaction can be excision, integration, inversion or translocation (Figure 4.1) (Sauer, 1993; Thorpe & Smith 1998; Oumard et al., 2006; Grindley et al., 2006; Ringrose et al., 1998; Boehm, 2000).

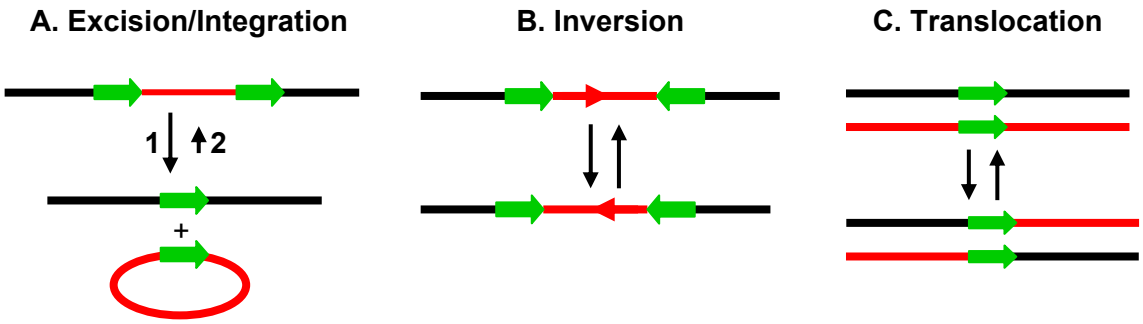

Figure 4.1. **The possible outcomes of site-specific recombination.** A. Two recombination sites in *cis* cause the excision of the DNA segment between them (1), as two recombination sites in *trans* can lead to integration (2), excision is favoured over integration if the two recombination sites are identical. B. Two recombination sites in opposite orientation cause the inversion of the DNA segment between them. C. translocation can occur if the two sites are on different chromosomes (adapted from Boehm, 2000).

The phage ΦC31-derived integrase, the bacteriophage P1-derived Cre, and the yeast-derived Flp are probably the best-characterised and widely used site-specific recombination systems in eukaryotes, certainly in insects.

The ΦC31 integrase, from the *Streptomyces* bacteriophage ΦC31, catalyses site-specific integration between two recognition sites: *attP* and *attB*. The *attP* and *attB* sites have different nucleotide sequence but both have a short identical recombination core (TTG). DNA strand exchange by the integrase occurs via a concerted, four-strand staggered break-and-rejoining mechanism, during which a phosphor-serine linkage is formed between the enzyme and the target DNA (Smith et al., 2004). Recombination between the two recombination sites results in the formation of two new hybrid sequences called *attL* and *attR*. When bound to the hybrid sites, the integrase is unable to obtain the correct conformation necessary for synapsis and therefore, in the absence of accessory factors, further recombination (excision) is prevented, making recombination unidirectional (Thorpe et al., 2000; Rowley et al 2008). The ΦC31 integrase has been shown to be functional in prokaryotic and eukaryotic cells (Thorpe & Smith 1998; Groth, 2000; Thomason et al., 2001; Khan et

al., 2005; Andreas et al., 2002; Thyagarajan et al., 2001; Allen & Weeks, 2005). In insects, it has been used to transform *D. melanogaster* (Groth et al., 2004; Fish et al., 2007), *Ae. aegypti* (Nimmo et al., 2006), medfly (Schetelig et al., 2009), *Ae. albopictus* (Labbé et al., 2010) and *Anopheles gambiae* (Meridth et al., 2011).

The Cre-*loxP* site-specific recombination system, of bacteriophage P1, consists of the Cre protein ("**C**auses **re**combination") and its cognate recombination site (Sadowski, 1993). The bacteriophage P1 is present in its host, *E. coli,* as a single circular DNA molecule. The Cre protein separates dimeric P1 DNA into single copies of P1 DNA during cell division, ensuring efficient segregation of the P1 plasmid (Austin et al., 1981; Sadowski, 1993). The canonical *loxP* ("**l**ocus **o**f crossing over (**x**) **P**1") site is 34 bp long and consists of two identical 13-bp inverted repeats, termed symmetrical elements, flanking an A-T rich, asymmetric 8-bp spacer region (Hoss & Abremski, 1998).

The Flp-*FRT* site-specific recombination system originates from the *Saccharomyces cerevisiae* 2 μm plasmid (Sadowski, 1995). *In vivo*, the Flp protein is involved in the amplification of the 2-μm plasmid (Sadowski, 1995; Grindley et al., 2006). The *FRT* site (**F**lp **R**ecognition **T**arget) is 48 bp long and consists of three symmetric elements 'a', 'b' and 'c' that surround an 8-bp spacer region. Symmetrical elements 'a' and 'b' are inverted repeats separated by an asymmetric 8-bp core region and nearly identical, only differing on one nucleotide. Elements 'b' and 'c' are directly orientated with respect to each other and are identical (Proteau et al., 1986; Huffman & Levene, 1999).

The Cre and Flp recombinase mode of action is remarkably similar. Both Cre and Flp bind to symmetric elements of their cognate *loxP* and *FRT* sites, respectively, with high affinity, and bring the sites together in a synaptic complex. During synapsis, both

enzymes catalyse cleavage of the DNA strands, in the 8-bp core region, through a nucleophilic attack of a tyrosine residue on the DNA backbone, and reciprocal exchange of the two DNA strands, with a Holliday junction intermediate (Zhu & Sadowski, 1995; Gronostajski & Sadowsoki, 1985). Furthermore, in both systems no accessory DNA or protein components are required to catalyse recombination (Sadowski, 1993). As *loxP* and *FRT* sites are restored after recombination, recombination is reversible in the presence of the recombinase (Baer & Bode, 2001). Since intra-molecular excision is kinetically favoured over bi-molecular integration, excision events exceed integration events (Kilby et al., 1993; Wimmer, 2005; Lyznik et al., 2007). In insects, Cre-*loxP* has been used to transform *D. melanogaster* (Siegal & Hartl, 1996; Oberstein et al 2005) and Cre recombinase has been shown to be functional in *Ae. aegypti* (Jasinskiene et al., 2003). Flp-mediated site-specific recombination has been successfully used in *D. melanogaster* (Golic & Lindsquist, 1989; Golic et al., 1997; Horn & Handler, 2005) and it has been shown to be functional in *Ae. aegypti* embryos (Morris et al., 1991). Both systems have also been shown to be active in other eukaryotic systems, such as yeast, plants and mammalian cells (Sauer, 1989; Sauer & Henderson 1988; Odell et al., 1990; Davies et al., 1999; Ludwig et al., 1998; Albert et al., 1995).

The use of site-specific recombinases is, potentially, a powerful tool for insect transgenesis. Cassettes, in a plasmid carrying a recombination site, could be repeatedly inserted, by the appropriate enzyme, into a pre-existing engineered genomic location containing the other recombination site. This site would be previously identified as particularly useful in terms of transgene expression levels, ability to make the insertion homozygous, and with low fitness costs to the insect.

Furthermore, a range of characterised insertion sites could be created each showing different levels of expression, to optimise expression of the gene cassette of interest.

Recombination-mediated cassette exchange

Recombinase-mediated cassette exchange (RMCE) is an integration strategy that makes use of site-specific recombinases, in which a donor sequence and a target sequence are both flanked by site-specific recombination sites. Double reciprocal recombination between the two sites results in the integration of the DNA segment between them as long as both sites are heterospecific (cannot recombine together). Cre- and Flp-RMCE has been used successfully used in mammalian cells (Lauth et al., 2002; Liu et al., 2007), plants (Nanto et al., 2007) and prokaryotes (Louwerse et al., 2007). In insects, Cre- and Flp-RMCE has been developed for *D. melanogaster* (Osberstein et al., 2005; Horn & Handler, 2005) and ΦC31-RMCE, using two inverted *attP* and two inverted *attB* sites, has also been achieved in *D. melanogaster* (Bateman et al., 2006).

Such a technique would be of great value as only desirable cassettes are inserted, in contrast to integration strategies that employ a single recombination site, where the entire donor plasmid is inserted, with associated prokaryotic sequences/antibiotic resistance genes. This would facilitate permission for field releases since transgenic insects for field release must to be free of drug-resistance sequences (Moreira et al., 2002), to prevent horizontal gene transfer. Furthermore, the insect line carrying the pre-existing docking site could be re-used for the insertion of new transgenes without the need to generate multiple lines.

Aims

Following the first successful transposon-mediated germline transformation of the diamondback moth (Chapter 1), this chapter focuses on targeted genomic integration using the ΦC31-integrase and the Cre and Flp recombinases, as a means to improve diamondback moth transgenesis. The combination of the ΦC31 integrase with the Cre and Flp recombinases, and Cre- and Flp-RMCE have also been investigated in order to further broaden the opportunities for targeted manipulation of the diamondback moth genome.

4.2 Materials and Methods

4.2.1. Generation of DNA constructs

4.2.1.1. OX4034

A 574 bp fragment, containing an *attB* sequence, was removed from construct OX3297, pB[*attB*-DsRed2-*tetO*21-tTAV], by digestion with *Kpn*I. The fragment was then inserted into construct OX1186, psc[Opie-nls-AmCyan-nls-SV403'UTR], previously digested with the same restriction enzyme, making OX4034, psc[*attB*-Opie-nls-AmCyan-nls-SV403'UTR].

4.2.1.2. OX4389

For further details see Chapter 3, section 3.2.1.1.

4.2.1.3. OX4433

Construct OX4433, psc[*attB*-Hr5ie1-nls-DsRed2-nls-SV403'UTR] was made by digesting construct OX4259, psc[*attB*-hr5*ie1*-ZsGreen-SV403'UTR], sequentially, with *Bgl*II/*Age*I to remove the ZsGreen fragment, which was replaced with a nls-DsRed2-nls fragment. The nls-DsRed2-nls fragment was removed from construct OX4204, pB[Aetopi-nls-DsRed2-nls-3×P3-DsRed-SV403'UTR], by sequential digestion with *Bgl*II and *Age*I.

4.2.1.4. OX4652

In order to make construct OX4652, two adaptors, consisting of a *loxP* and a *FRT* site each, were inserted into OX4573, pB[Hr5*ie1*-DsRed-nls-Sv403'UTR]. Construct OX4573 was digested with *Not*I/*Spe*I and ligated to the *Not*I/*Spe*I *loxP* adaptor. The resulting construct was then digested with *Pac*I/*Avr*II ligated to the *Pac*I/*Avr*II *FRT* adaptor, creating OX4652, pB[*loxP*-Hr5*ie1*-nls-DsRed2-nls-SV403'UTR-*FRT*]. Adptors *Not*I/*Spe*I *loxP* and *Pac*I/*Avr*II *FRT* were made using oligonucleotides L1-F and L1-R and L2-F and L2-R, respectively. Oligonucleotides can be found in appendix 3.

4.2.1.5. OX4655

Construct OX4655, pB[*FRT*-*loxP*-Hr5*ie1*-nls-DsRed2-nls-*FRT3*-*lox2272*] was made by introducing four adaptors consisting of two heterologous *lox* sites and two heterologous *FRT* sites into construct OX4573, pB[Hr5*ie1*-DsRed-nls-Sv403'UTR]. Construct OX4573 was digested with *Not*I/*Spe*I and ligated to the *Not*I/*Asc*I *FRT* and AscI/SpeI *loxP* adaptors. The resulting construct was then digested with *Pac*I/*Avr*II ligated to the *Pac*I/*Xma*I *FRT3* and *Xma*I/*Avr*II *lox2272* adaptor. Adaptors *Not*I/*Asc*I *FRT*, *Asc*I/*Spe*I *loxP*, *Pac*I/*Xma*I *FRT3* and *Xma*I/*Avr*II *lox2272* were made using oligonucleotides L3-F and L3-R, L4-F and L4-R, L5-F and L5-R and L6-F and L6-R, respectively. Adjacent adaptors were ligated together before ligation with the vector backbone. Oligonucleotides can be found in appendix 3.

4.2.1.6. OX4663

The OX4663 construct was made by inserting two adaptors, consisting of a loxP and a FRT site each, inserted into OX4661 Pscript[Opie2-ZsGreen-nls-SV40]. Construct OX4661 was digested with *AvrII/SphI* and ligated to the *AvrII/SphI* loxP adaptor. The resulting construct was then digested with *NdeI/AscI* and ligated to the *NdeI/AscI* FRT adaptor. Adaptors *AvrII/SphI* loxP and *NdeI/AscI* FRT were made using oligonucleotides L7-F and L7-R and L8-F and L8-R, respectively. Oligonucleotides can be found in appendix 3.

4.2.1.7. OX4673

Construct OX4664 was made by introducing four adaptors consisting of two heterologous loxP sites and two heterologous FRT sites into construct OX4661 Pscript[Opie2-ZsGreen-nls-SV40]. Construct OX4661 was digested with *AvrII/SphI* and ligated to the *AvrII/SexAiI* FRT and *SexAI/SphI* loxP adaptors. The resulting construct was then digested with *NdeI/AscI* ligated to the *NdeI/SexAI* FRT 3 and *SexAI/AscI* loxP2272 adaptor. Adaptors *AvrII/SexAiI* FRT, *SexAI/SphI* loxP, *NdeI/SexAI* FRT 3 and *SexAI/AscI* loxP2272 were made using oligonucleotides L9-F and L9-R, L10-F and L10-R, L11-F and L11-R and L12-F and L12-R, respectively. Adjacent adaptors were ligated together before ligation with the backbone vector. Oligonucleotides can be found in appendix 3.

4.2.1.8. Other constructs used in this study

Constructs used in this study which were made by colleagues:

OX3441: pB[*attP*-Hr5*ie1*-DsRed-nls-SV40], madeby Dr Guoliang Fu

OX4540: pB[FRT-*loxP*-*Opie2*-DsRed-nls-SV40-attP], made by Dr Sarah Scaife

OX4580: psc[attB-*FRT*-*loxP*-Hr5*ie1*-ZsGreen-nls-SV40], madeby Dr Sarah Scaife

4.3. Results

4.3.1. ΦC31 integrase-mediated site-specific integration

4.3.1.1. ΦC31 integrase-mediated integration of OX4034 into OX3441 strains

To study the applicability of ΦC31 integrase in diamondback moth transformation, genomic docking sites were created using construct OX3441 (Figure 4.2). This construct, with two transposable *piggyBac* pairs at each end (for potential excision to render the insect transposon-free (Dafa'alla et al., 2006)), consisted of an *attP* recognition site for ΦC31 integrase and a Hr5*ie1*-DsRed2 transformation marker flanked by two nuclear localisation signals. The construct was used to transform diamondback moth using *piggyBac* (see Chapter 3 for further details).

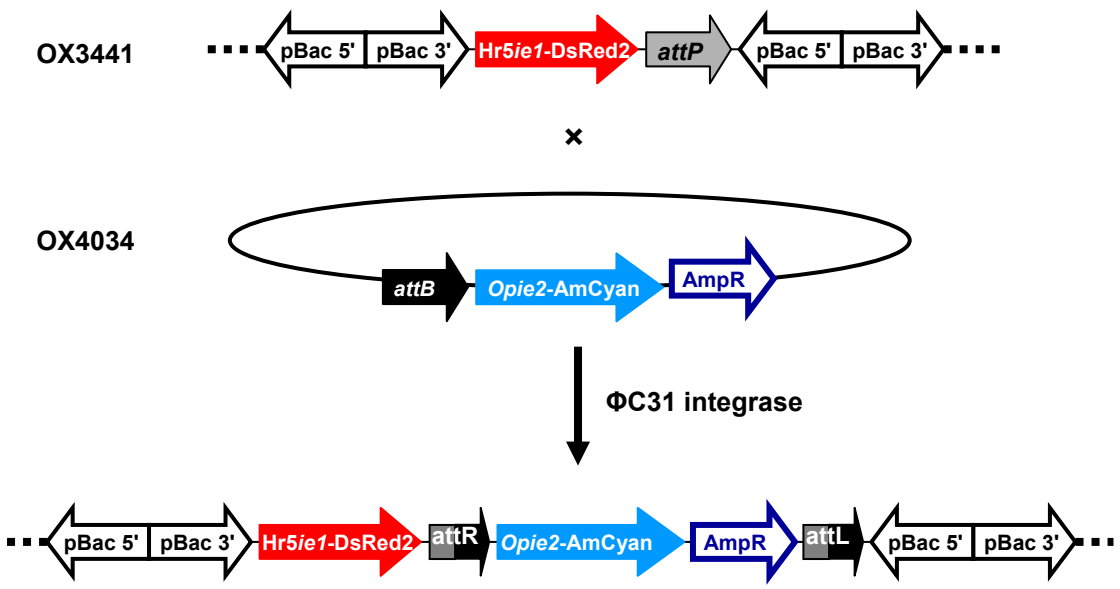

Figure 4.2. Schematic representation of constructs OX3442 and OX4034, and resulting integration product.

Eggs from each OX3441 line (A, C and H) were injected within 1 h of being laid. The injection mix consisted of OX4034 plasmid and wild-type ΦC31 integrase mRNA, at concentrations of 350 ng/µl and 500 ng/µl, respectively, in injection buffer (Table 4.1). OX4034 is a "marker-only construct" carrying an *attB* ΦC31 integrase recognition site, an *Opie2*-AmCyan transformation marker and two nuclear localisation signals flanking the fluorescent protein (Figure 4.2.). Between 1023 and 1239 embryos were injected for each line (Table 4.2). Of these, 286-312 (28%-32%) survived to pupation. Injection survivors were crossed to wild-type mates in pools at a ratio of 1♂:3♀, and their progeny were screened for fluorescence. Successful integration would have been identified as insects expressing both DsRed2 and AmCyan fluorescent proteins (Figure 4.2). However, none of the screened pupae showed any AmCyan expression and, therefore, no integration events were detected for any of the lines.

Table. 4.1. Data for injections of OX4034 into OX3441 lines.

ΦC31 integrase	No. embryos injected	Total no. injection survivors (G_0s)		No. integration events
		Transgenic	Wild-type	
OX3441A	1023	171	115	0
OX3441C	1239	271	127	0
OX3441H	1115	213	121	0

Site-specific integration events were not detected in any of the OX3441 lines tested. As site-specific integration mediated by the ΦC31 integrase has been shown to work in a wide range of organisms, it was expected to work in diamondback moth. As site-

specific integration did not occur, there was a possibility that the integrase was defective. PCR amplification of the integrase during cloning might have introduced a mutation that rendered the integrase inactive. The original plasmid containing the integrase sequence was sent for sequencing. Sequencing results showed the integrase sequence was correct. In addition, the attP sequence was also sequenced confirming the integrity of the site and a sample of each injection mix was always run on an agarose gel before injection to ensure that the integrase mRNA was not degraded.

Frequency of integration depends on the DNA sequence of the site and the chromosomal context of the receptor (*attP*-carrying transgene) which may influence gene expression and integrase access (Thyagaranjan et al., 2001). Sites buried in inaccessible chromatin structures, for instance, might not be easily accessed by the integrase, affecting synapsis formation. If the *attP* site is inserted in a region of compact DNA there might not be enough space for the integrase dimers to reach the *attP* and bring it close to the *attB* site in a synapsis. However, three independent lines were available for testing, increasing the chance of avoiding this potential problem. The OX3441 construct was compared to other constructs used by colleagues, with and without success, in ΦC31 site-specific transformation of *Ae. aegypti* (Nimmo et al., 2006), *Ae. albopictus* (Labbé et al., 2010), medfly (personal communication, Thomas Ant) and pink bollworm (carried out by myself). Comparison of the construct maps showed that, in all constructs that failed (integration was unsuccessful), the *attP* was immediately preceded by either a SV40 or Hr5 sequence, both of which are highly repetitive sequences and have their own secondary structure. All the constructs that worked (permitted site-specific integration) were based on that used by Nimmo et al., (2006) and all contain a 307 bp segment of incomplete *piggyBac* transposase sequence (a relic of construct cloning) that, in this context, was perhaps acting as a

spacer between the *attP* and other construct components. It is possible that the composition of DNA flanking the *attP* site is important for function of the integration reaction. This sequence may, for example, affect the correct positioning of the integrase in relation to the *attP* site, preventing it from establishing transient covalent attachment and inhibiting synapsis. Only conformations that permit stable synapsis result in site-specific integration. Formation of an incorrect synapse between *att* sites inhibits recombination, possibly due to mismatches in the crossover sequence (Smith et al., 2004). Considering these facts, new *attP* and *attB*-carrying constructs as similar as possible to those used by Nimmo et al. (2006), were built for future experiments.

4.3.1.2. ΦC31 integrase-mediated integration of OX4433 into OX4389 strains

A new construct, OX4389, containing the original *attP* site flanked by DNA sequences from the original construct from Nimmo et al. (2006) and the *Opie2*-ZsGreen transformation marker, was built in order to generate *attP* acceptor lines (Figure 4.3). Although, *attP* sequences as short as 39 bp (Groth et al., 2000) have been shown to be functional in other organisms such as *E. coli* (84 bp) (Thorpe & Smith, 1998) and medfly (52 bp) (Schetelig et al., 2009), a longer 221-bp *attP* has been used in OX4389, and all of the constructs described in this chapter. A longer *attP* sequence has been shown to work in *D. melanogaster* and humans (Bischof et al., 2007; Groth et al., 2000) and is thought to increase the chances of integration compared to a shorter sequence. OX4389 construct details and injection data can be found in Chapter 3 (section 3.3.1).

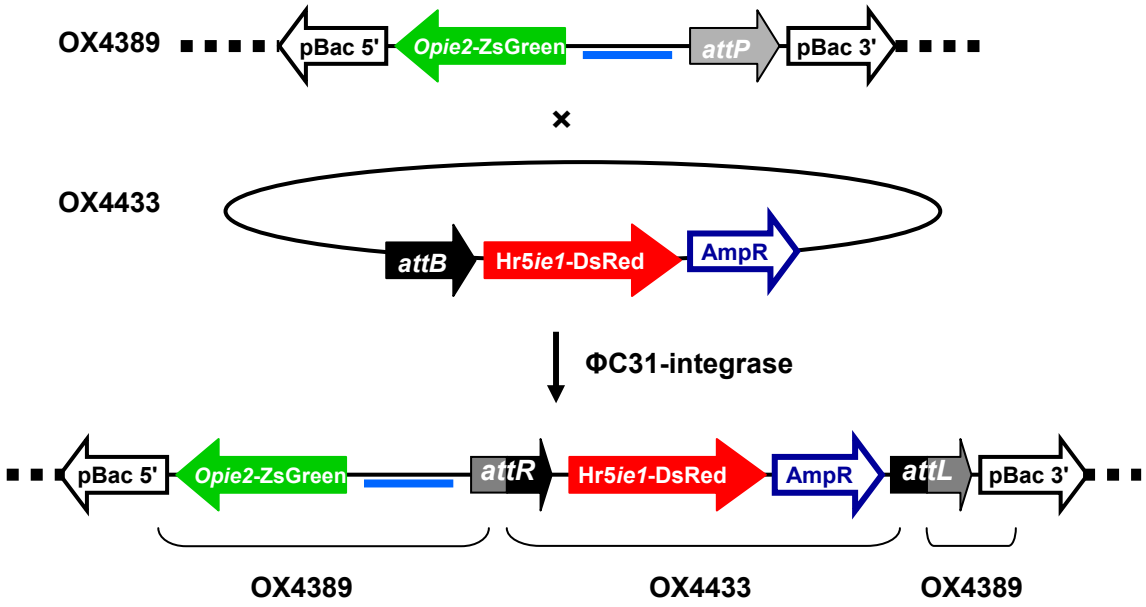

Figure 4.3. Schematic representation of OX4389 and OX4433 constructs, and of the final ΦC31 integration product. Blue line represents the incomplete, relic 307 bp *piggyBac* transposase sequence between the *attP* site and the marker gene. In total, 634 bp separate the *attP* from the marker gene. This distance is approximately 2.5× higher than the distance between these sequences in OX3441.

Embryos from OX4389A, B and C were injected with OX4433 donor plasmid and ΦC31 mRNA (Figure 4.3, Table.4.3). OX4433 carries a 285-bp *attB* site, previously shown to work in *D. melanogaster* by Bischof and colleagues (2007), to integrate into *attP* site and a Hr5-*ie1*-DsRed transformation marker, with two nuclear localisation signals flanking the fluorescent protein. For all lines, the injected embryos were derived from a cross of fluorescent individuals and therefore comprised a mixture of hemizygotes (with one copy of the transgene and so one *attP* site), homozygotes (with two copies of the transgene and so two *attP* sites) and wild-type. Wild-type injection survivors lacking the *Opie2*-ZsGreen marker from construct OX4389 were discarded as they did not carry the *attP* site. As previously, injection survivors, carrying the *Opie2*-ZsGreen marker, were crossed to wild-type mates, in pools of 2-5 transgenics,

and their progeny screened for fluorescence. Successful integration was identified as insects expressing *Opi2*-ZsGreen and red Hr5*ie1*-DsRed2 fluorescence (Figure 4.2).

Table. 4.2. ΦC31-mediated site-specific integration injection data for OX4433 into OX4389.

Line	No. embryos injected	Total no. injection survivors (G0)		No. integration events	Efficiency (%)
		Transgenic Go´s	Wild-type Go´s		
OX4389A	2061	406	217	1	0.25
OX4389B	1339	343	178	2	0.58
OX4389C	1256	414	177	0	-

For OX4389A, 2061 embryos were injected, of which 623 (30.2%) survived to pupae (Table 4.2). Of the injection survivors, 406 individuals expressed the green fluorescence in the body and therefore carried at least one copy of OX4389A. These were crossed to wild-type of the opposite sex in a ratio of 1♂:3♀, in pools of 2-5 G_0 individuals. Thirteen transgenics were recovered from one cross, out of a total of 81 G_0 crosses, and used to found line OX4433[4389A]. This corresponds to a minimum transformation efficiency of 0.25% if all the G_0´s were fertile. This assumes that all the transgenic individuals are derived from a single transformation event and one G_0 parent. This may well be an underestimate; however, since all the integration events occur into the same docking site, so independent events within one pool cannot be distinguished.

A total of 1339 OX4389B embryos were injected. Of the injected animals, 521 (38.9%) matured to pupation. Of these, 343 expressed green fluorescence and were

crossed to wild-type mates, as in OX4389A. Out of 69 crosses, two produced transgenic offspring. Although these transgenics are likely to be the same line, for future analysis of integration events, they were kept separately as sub-lines OX4433[4389B]-1 and OX4433[4389B]-2. The minimum calculated integration efficiency is 0.58%.

For line OX4389C, 1256 embryos were injected and 591 (47.1%) survived to pupation. As for the previous lines, a total of 414 G_0 individuals expressing green fluorescence were crossed to wild-type mates. A total of 83 crosses were set up but no integration events were detected.

In all OX4389 lines, the site-specific integration of Hr5*ie1*-DsRed showed the same expression pattern as the *Opie2*-ZsGreen of the parental OX4389 lines, as expected, since both markers are being influenced by the same neighbouring genomic DNA. This was also observed in site-specific integration in *Ae. aegypti* (Nimmo et al., 2006) and *Ae. albopictus* (Labbé et al., 2010). No difference in fluorescence intensity or pattern was observed between the new integrated lines (Figure 4.4).

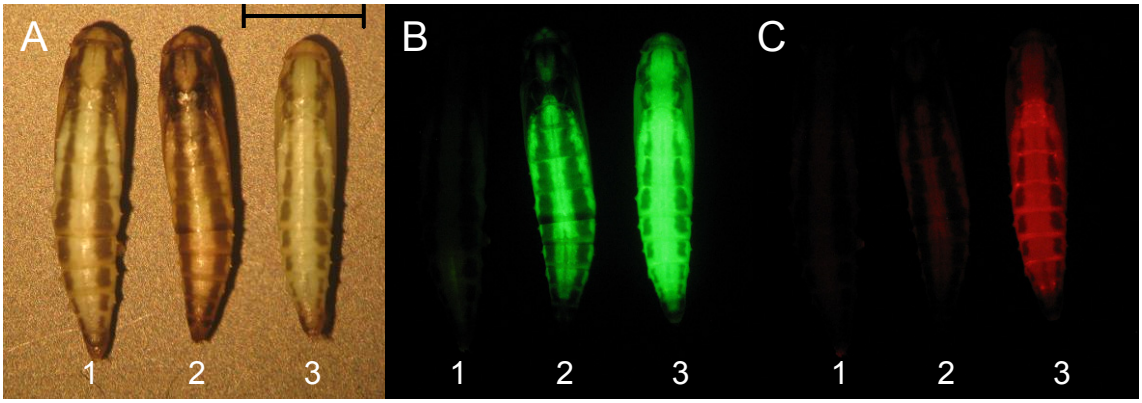

Figure 4.4. Marker expression (pupae, dorsal view) of transgenic diamondback moth OX4389A with and without ΦC31-mediated site-specific integration of the OX4433 construct. Photographs illustrating pupae under (A) white-light, (B) green excitation light and filters and (C) red excitation light and filters. Pupa 1 is wild-type; pupa 2 is OX4389A, the parental line carrying only the *Opie2*-ZsGreen marker; and pupa 3 is OX4433[4389A], the integrated line carrying both *Opie2*-ZsGreen and Hr5*ie1*-DsRed markers. The remaining OX4433[4389B]-1 and OX4433[4389B]-2 lines show the same green and red fluorescent profile as OX4433[4389A] (data not shown). Scale bar represents 2.5 mm.

PCR analysis of site-specific integration

The ΦC31-integrase sometimes recognises pseudo-*attP* sites, which are genomic sequences with some similarity to the *attP* sequence. These are called pseudo-integrations (Thygarajan et al., 2001, Nimmo et al., 2006). As the pseudo-sites are not identical to *attP* sequence, pseudo-integration is imprecise and less frequent than true site-specific integration. In order to determine whether the presence of both markers in the generated transgenic individuals was due to a true integration event or a pseudo-integration, genotyping PCRs were conducted on the three lines generated. PCR amplification of single-pupa genomic DNA was performed using the primer pairs 'Int2' and 'Int3' for the *attL* junction (expected amplified fragment size, 425 bp), and 'Int1' and 'Int4' for the *attR* junction (expected amplified fragment size, 380 bp) (primer sequences can be found in appendix 3). Both primer pairs flank the *att* sites, so should only result in amplification if a true integration event has occurred.

PCR confirmed the insertion of the OX4433 donor plasmid into the *attP* sites of the OX 4389 construct, for lines OX4389A and B (Figure 4.5 and appendix 5). The *attL* and *attR* junctions should only be present in integrated gDNA. The recipient line promoter *Opie2* should be present in integrated and recipient line gDNA. Sequences *ie1* and AmpR should only be present in the integrated gDNA, as they come from the donor plasmid. The *attP* sequence should only be present in the recipient line gDNA

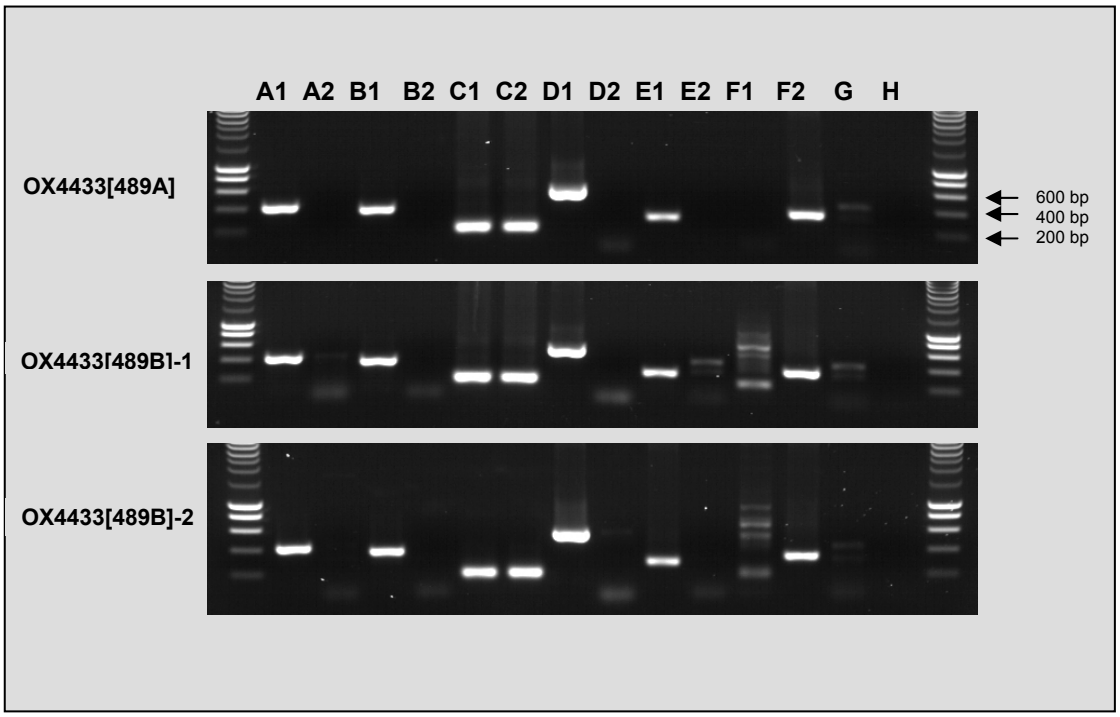

Figure 4.5. Molecular confirmation of site-specific integration. PCR was used to verify the presence of the expected junction fragments in the integrants. In all panels, lane numbers 1 and 2 indicate PCR performed on gDNA from integrants and from recipient OX4389 line individuals (used as control), respectively. The letters A-H represent the expected bands for: A, *attL* (425 bp); B, *attR* (380 bp); C, *Opie2* (202 bp); D, *ie1* (555 bp); E, AmpR (250 bp); F, *attP* (345 bp); G, wild-type control, primer set *attR* (380 bp); and H, no gDNA control, primer set *attR* (380 bp). Smart ladder is run as a marker at both ends.

The canonical *attP-attB* recombination was also verified by sequencing the *attL* and *attR* PCR fragments (Table 4.3). Note that the sequences obtained for all lines were exactly the same, as they all contain the same two constructs.

Table. 4.3. Sequence analysis of the *attL* and *attR* junctions. Sequences in red and green show the OX4433 and OX4389 sequences, respectively, flanking the *att* junction (in black). Underlined sequences correspond to the primer sequences used to amplify the junction. TTG(bold underlined) represents the recombination core.

attL	CTGCAAGGCGATTAAGTTGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGC CAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGT CGACGATGTAGGTCACGGTCTCGAAGCCGCGGTGCGGGTGCCAGGGCGTGCCC**TTG**AGTTCTC TCAGTTGGGGGCGTAGGGCCGCCGACATGACACAAGGGGTTGTGACCGGGGTGGACACGTACG CGGGTGCTTACGACCGTCAGTCGCGCGAGCGCGACTAGTTCTAGAGCGGCCGCCTGCAGTAGG AAGACGAATAGGTGGCCTATGGCATTATTGTACGGAATGATAAACATTGCCTGCATAAATTCT TTTATTATATACAGCCATAATGTCAGTAGCAAGGGAGA
attR	AGTGGACACGCTAGACCAAATGTGTTCTGTGATGACCTGCAGCCCGGGGGATCCACTAGTACT GACGGACACACCGAAGCCCCGGCGGCAACCCTCAGCGGATGCCCCGGGGCTTCACGTTTTCCC AGGTCAGAAGCGGTTTTCGGGAGTAGTGCCCCAACTGGGGTAACCT**TTG**GGCTCCCCGGGCGC GTACTCCACCTCACCCATCTGGTCCATCATGATGAACGGGTCGAGGTGGCGGTAGTTGATCCC GGCGAACGCGCGGCGCACCGGGAAGCCCTCGCCCTCGAAACCGCTGGGCGCGGTGGTCACGGT GAGCACGGGACGTGCGACGGCGTCGGCGGGTGCGGATACGCGGGGCAGCGTCAGCGGGTTCTC GACGGTCACGGCGGGCATGTCGACGGTATCGATAAGCTTAAGTCATTTGTTTTTAAAATTGAA CTGGCTTTACGAGTAGAATTCTACGCGTAAAACACAATCAAGTATGAGTCATAAGCTGATGTC ATGTTTTGCACACGGCTCATAACCGAACTG

Transgene-hemizygous individuals were crossed to wild-type mates and, in generation 3, the number of transgenic and wild-type progeny was counted (Table 4.4). For each line, progeny phenotype data show Mendelian inheritance: the number of transgenic and wild-type individuals was not significantly different from the expected 50:50 ratio (transgenic:wild-type). This is consistent with each line carrying a single insertion. All lines were reared for several generations and no segregation of the two markers was observed, indicating that integration is stable. Development time from egg to adulthood of transgenic individuals was similar to that of wild-type individuals, approximately 21 days in our laboratory conditions. From these observations, site-specific integration events were shown not to affect diamondback moth viability.

Table 4.4. Mendelian inheritance of the transgene in 4433[4389] lines. Ratio of transgenic versus wild-type. χ^2 test were p>0.05 represents no significant difference between the transgenic:wild-type ratio obtained and the expected 50:50 ratio.

Line	4433[4389A]	4433[4389B]-1	4433[4389B]-2
Transgenic	160	201	189
Wild-type	173	183	196
χ^2 (d.f=1)	0.508	0.844	0.127
p value	0.4762	0.3583	0.7213

Flanking analysis of the 4433[4389] lines revealed that the OX4389 recipient construct insertion site remained the same after integration of the donor plasmid, indicating that (as expected) *piggyBac* did not remobilise. Sequencing of the insertion sites of lines 4433[4389B]-1 and 4433[4389B]-2 showed them to be the same, as expected, so they were pooled to become 4433[4389B] (data not shown).

4.3.2. Optimised ΦC31-integrase

In an attempt to increase ΦC31-integrase-mediated integration efficiency, two modified versions of the ΦC31-integrase were tested. One version, OX4505, consists of the wild-type ΦC31-integrase codon-optimised for three insect species: *D. melanogaster*, *Ae. aegypti* and *B. mori*. The other version, OX4533 consists of the optimised ΦC31-integrase with a nuclear localisation sequence (NLS) attached to its N- and C-termini. Previous reports show that the addition of a NLS to the C-terminal end of the integrase substantially enhances integration frequency (Andreas et al., 2002; Bischof et al., 2007). Both modified ΦC31-integrases were used, as capped

mRNA, to mediate integration of the OX4433 donor plasmid into OX4389A and B recipient lines, shown to work previously (as detailed above) with the wild-type ΦC31-integrase. Pre-blastoderm diamondback moth embryos from OX4389A and OX4389B were injected with OX4433 plasmid and capped mRNA of either OX4505 or OX4533, at a concentration of 350 ng/µl of donor plasmid and 500 ng/µl of mRNA (Table.4.5).

Table. 4.5. Injection data for OX4433 into OX4389 lines with modified ΦC31 integrases

ΦC31 integrase	Line	No. embryos injected	Total no. injection survivors (G_0s)		No. integration events
			Transgenic	Wild-type	
OX4505	OX4389A	1128	76	40	0
	OX4389B	1256	112	63	0
OX4533	OX4389A	1179	271	130	0
	OX4389B	1077	295	138	0

A total of 1128 and 1256 embryos of OX4389A and OX4389B, respectively, were co-injected with OX4433 donor plasmid and optimised ΦC31-integrase capped mRNA (OX4505). Of these, 10.3% (116) for OX4389A and 13.4% (175) for OX4389B survived to pupation. The survival rates, for both lines (10-13%), were very similar, but low compared to those seen in previous experiments, where 30-47% survival had been achieved (Table 4.5). Wild-type injection survivors were discarded and the remaining transgenic survivors were crossed in pools with wild-type mates. Progeny were screened for DsRed expression, the OX4433 marker. DsRed expression was not detected in any pupae.

A total of 1179 and 1077 OX4389A and OX4389B embryos, respectively, were co-injected with OX4533 donor plasmid and optimised ΦC31-integrase mRNA, flanked by two NLS (OX4533). Survival rates of 34.0% (401) for line OX4389A and 40.2% (433) for line OX4389B were obtained. As previously, wild-type survivors were discarded, as they do not carry the *attP* docking site, and the transgenic survivors, carrying the *attP* site were crossed to wild-type. No DsRed expression was detected in the screened pupae. Considering that experiments were carried out on multiple days, using multiple injections mixes made with different DNA and mRNA preps, and that integration has been previously achieved in OX4389A and B lines, it is likely that the optimisation of the ΦC31-integrase rendered the enzyme less efficient. As integration had been achieved with wild-type ΦC31-integrase (section 4.3.1.1), no further work was carried out with the optimised ΦC31-integrases.

4.3.3. Transgene ΦC31-mediated integration with subsequent Cre- and Flp-mediated excision of DNA segments.

A weakness of the ΦC31-integrase system is that the entire donor plasmid is integrated, including antibiotic resistance genes and bacterial sequences. In order to test whether these sequences could be removed post-integration, new recipient and donor constructs were built[4] carrying a marker gene, and an *attP* and *attB* site, respectively. In addition to the *att* sites, both constructs carried a *loxP* and a *FRT* site each, so that after integration of the donor plasmid into the recipient line, the segment of the DNA flanked by these two sites could be excised by the Cre or Flp recombinases. If Cre- and/or Flp-recombinase were active in the germline, excision of

[4] Constructs made by Dr Sarah Scaife

the transgene segment, which comprises the recipient construct's marker gene and the plasmid backbone (including ampicillin resistance gene, AmpR) from the donor plasmid, would result in progeny expressing only the donor plasmid marker gene (Figure4.6).

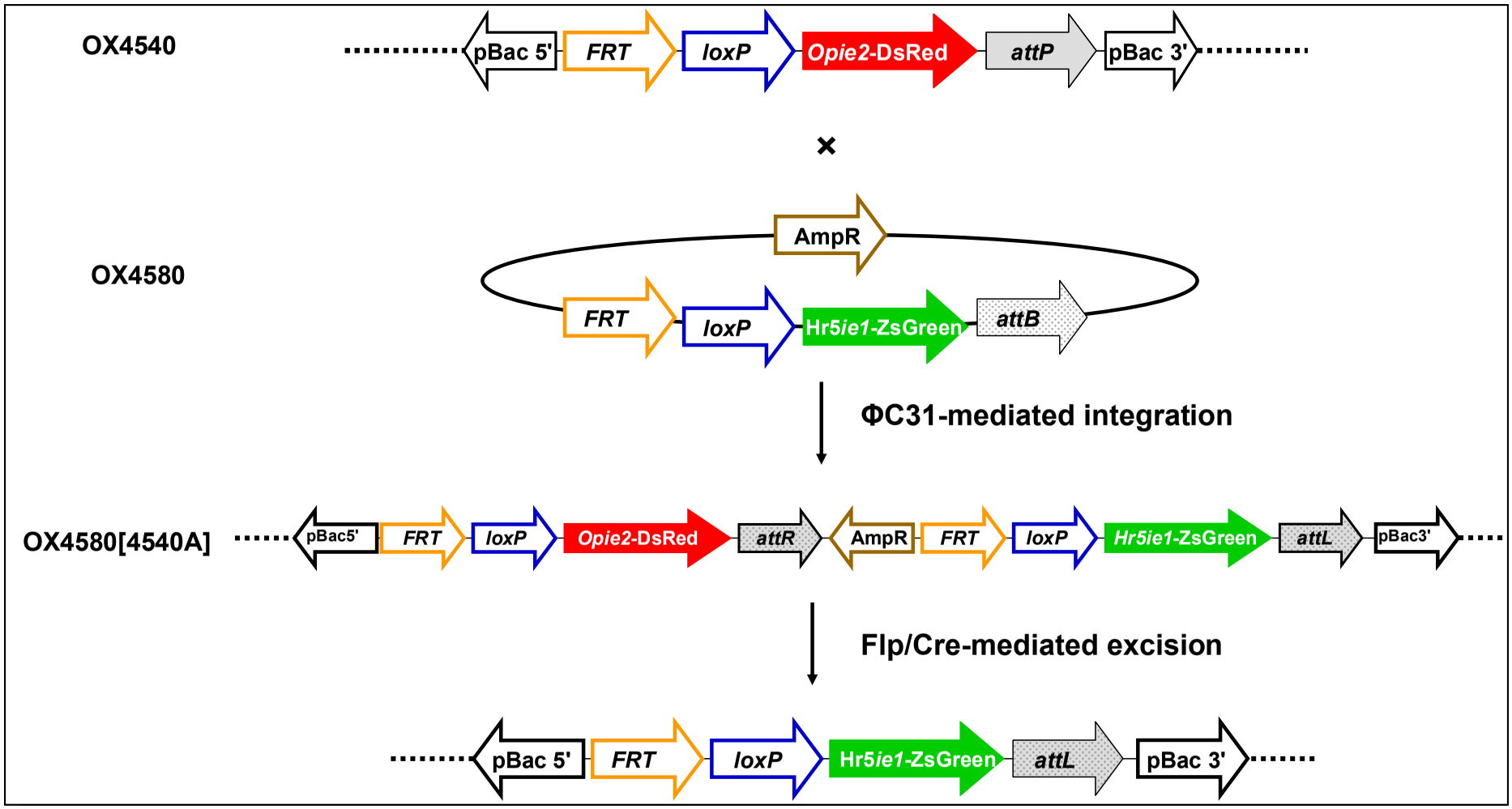

Figure 4.6. Schematic representation of OX4540 and OX4580 constructs, of the final ΦC31 integration product OX4580[4540A] and post-integration excision by Cre or Flp-mediated recombination.

4.3.3.1. Generation of OX4540 recipient lines in diamondback moth

The recipient construct, OX4540, was used to transform diamondback moth by *piggyBac*-mediated transformation. The OX4540 consists of a marker gene, *Opie2*-DsRed, a *loxP* site, a *FRT* site and an *attP* site, flanked by piggyBac terminal repeats (Figure 4.6). A total of 1868 wild-type embryos were co-injected with a transposase source (OX265). Of these, 873 (46.7%) survived to pupation and were crossed to wild-type mates in pools of five injection survivors. Progeny was screened for DsRed expression and 22 out of 175 crosses resulted in transgenics. Assuming 100% fertility of injection survivors, the minimum calculated integration efficiency is 2.5%.

Transgenic individuals of each cross were used to set up lines OX4540A-W. Mendelian inheritance data, collected on generation 2 (appendix 6), showed that lines OX4540C, K, N, O, and Q possibly carried multiple insertions of the transgene, as there was a significantly higher proportion of transgenic progeny compared to wild-type progeny (OX4540C, χ^2(d.f.=1)=10.588, p=0.0011; OX4540K, χ^2(d.f.=1)=7.446, p=0.0064; OX4540N, χ^2(d.f.=1)=28.000, p=0.0001; OX4540Q, χ^2(d.f=1)=8.000, p=0.0047; OX4540T, χ^2(d.f=1)=3.904, p=0.0482). For lines OX4540H (χ^2(d.f.=1)=13.433, p=0.0002) and OX4540Q (χ^2(d.f=1)=8.000, p=0.0047) the proportion of transgenic progeny was significantly lower than wild-type progeny which may indicate a fitness cost associated with the transgene. In all of the remaining lines, there was no significant difference between the proportion of transgenic and wild-type progeny, indicating that these lines carry a single transgene insertion, in a non-essential part of the genome. Although several lines seemed to carry multiple insertions, no obvious variation in the *Opie2*-DsRed marker expression intensity or pattern was observed.

4.3.3.2. ΦC31-integrase-mediated transformation of OX4540A strain

OX4540A, apparently carrying a single transgene insertion, was selected as the recipient line. The donor plasmid OX4580 carries a Hr5*ie1*-ZsGreen marker, a *loxP* site, a *FRT* site and an *attB* site to integrate into *attP*. Hemizygous OX4540A individuals were crossed to each other and their progeny, a mixture of hemizygote, homozygote and wild-type embryos, co-injected with capped ΦC31-integrase mRNA. A total of 1082 embryos from the recipient line were injected and, of these, 453 (41.9%) survived to pupation. Wild-type progeny (121 individuals out of 453) were discarded as they did not have an *attP* docking site. The remaining transgenic survivors (332 individulas ou of 453) were mated to wild-type and their progeny were screened for ZsGreen expression (the donor plasmid marker). Out of 91 crosses, one generated individuals expressing ZsGreen (Figure 4.7). These were used to found line OX4580[4540A]. The minimum calculated integration efficiency is 0.30%, which is similar to that obtained for OX4389A and B lines, 0.16% and 0.39%, respectively (Table. 4.3).

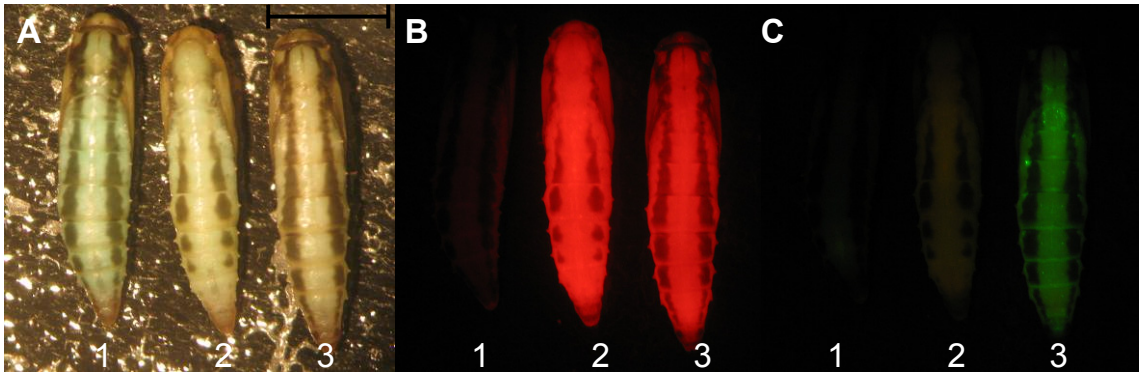

Figure 4.7. OX4580[4540A] fluorescence phenotype (pupae, dorsal view). (A) white light; (B) red excitation light and filters; and (C) green excitation light and filters. Numbers indicate (1) wild-type pupa; (2) recipient OX4540 line pupa and (3) OX4580[4540A] pupa. Bar scale size=2.5 mm.

ΦC31 integrase-mediated integration was confirmed by PCR (Figure 4.8). PCR amplification of singe-pupa genomic DNA with primer pairs amplifying across the *attL* and *attR* junctions (as before) were used to confirm site-specific recombination in pupae selected by phenotypic assay. Several controls were included such as the presence/absence of *Opie2* promoter sequence, *ei1* promoter, AmpR sequence and *attP* sequence. As a control, PCRs *attL* and *attR* junctions were also performed on the recipient OX4540 line genomic DNA. As expected, the recipient line promoter was present in integrated and recipient line gDNA. Sequences *ie1* and AmpR were only present in the integrated gDNA, as they belong to the donor plasmid. The *attP* sequence was only present in the recipient line gDNA.

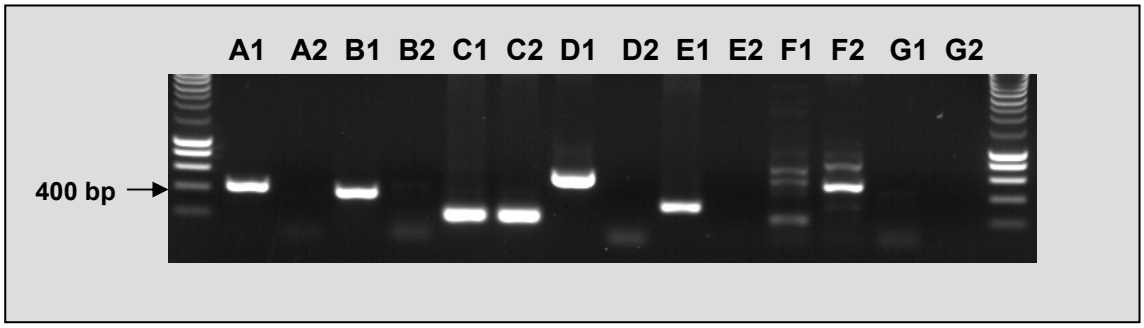

Figure 4.8. ΦC31-mediated site-specific integration of the OX4580 into OX4540A line of diamondback moth. Numbers 1 and 2 indicate PCR was conducted on gDNA from integrants and from recipient OX4540 line individuals, respectively. The letters A-H represent product bands for: A, *attL* (425 bp); B, *attR* (380 bp); C, *Opie2* (202 bp); D, *ie1* (555 bp), E, AmpR (250 bp); F, *attP* (345 bp); G, wild-type control (primer set *attL*); and H, no gDNA control (primer set *attL*). Smart ladder is run at both ends.

In addition, the integration event was confirmed by nucleotide sequencing of cloned amplification products (Table 4.6).

Table. 4.6. Sequence analysis of the *attL* and *attR* junctions. Sequence in Red and green show the OX4580 and OX4540 sequences, respectively flanking the *att* junction (in black). Underlined sequences correspond to the primer sequences used to amplify the junction. TTG (underlined, bold) represents the recombination core.

attL	CTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAAC GACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCC CCCCTCGAGGTCGACGATGTAGGTCACGGTCTCGAAGCCGCGGTGCGGGTGCCAGGGC GTGCCC**TTG**AGTTCTCTCAGTTGGGGGCGTAGGGCCGCCGACATGACACAAGGGGTTG TGGCCGGGGTGGACACGTACGCGGGTGCTTACGACCGTCAGTCGCGCGAGCGCGACTA GTTCTAGAGCGGCCGCCTGCAGTAGGAAGACGAATAGGTGGCCTATGGCATTATTGTA CGGAATGATAAACATTGCCTGCATAAATTCTTTTATTATATACAGCCATAATGTCAGT AGCAAGGGAGA
attR	ACACGCTAGACCAAATGTGTTCTGTGATGACCTGCAGCCCGGGGGATCCACTAGTACT GACGGACACACCGAAGCCCCGGCGGCAACCCTCAGCGGATGCCCCGGGGCTTCACGTT TTCCCAGGTCAGAAGCGGTTTTCGGGAGTAGTGCCCCAACTGGGGTAACCT**TTG**GGCT CCCCGGGCGCGTACTCCACCTCACCCATCTGGTCCATCATGATGAACGGGTCGAGGTG GCGGTAGTTGATCCCGGCGAACGCGCGGCGCACCGGGAAGCCCTCGCCCTCGAAACCG CTGGGCGCGGTGGTCACGGTGAGCACGGGACGTGCGACGGCGTCGGCGGGTGCGGATA CGCGGGGCAGCGTCAGCGGGTTCTCGACGGTC

4.3.3.3. Cre-induced germline excision

A total of 1206 OX4580[4540A] embryos, a mixture of hemizygotes, homozygotes and wild-type, were microinjected with 700 ng/µl of Cre recombinase capped mRNA. A total of 365 (30.3%) survived to pupation. Of these, 92 were wild-type and, therefore discarded; 269 showed patchy *Opie2*-DsRed marker expression intensity and patterns, and four completely lost the *Opie2*-DsRed marker expression. Transgenic survivors were crossed, in pools of 2-5 individuals, to wild-type mates. All progeny were checked for loss of the *Opie2*-DsRed marker expression. A total of 84 crosses were set up. One family did not produce any progeny, 75 crosses out of the remaining 83 (90.4%) fertile families produced some progeny expressing only marker Hr5*ie1*-ZsGreen (Figure 4.9).

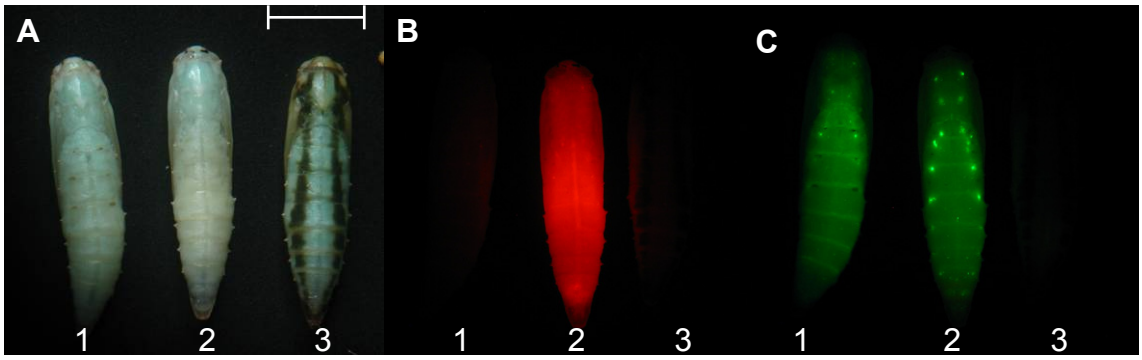

Figure 4.9. Cre-mediated excision fluorescent phenotype (pupae, dorsal view). (A) white light; (B) red excitation light and filters; and (C) green excitation light and filters. Numbers indicate (1) pupa expressing the post-excision phenotype; (2) parental OX4580[OX4540A] pupa and (3) wild-type pupa. Scale bar represents 3 mm.

Further analysis of the number of progeny, in each family, expressing only the Hr5*ie1*-ZsGreen marker revealed that the excision efficiency within a family could be as low as 0.88% and as high as 100% (raw data can be found in appendix 7).

To confirm the putative excision event, PCR amplification was performed on single-pupa genomic DNA. Primers were designed for the OX4540 and OX4580 sequence flanking the putative excised region so that a PCR product of 801 bp would only be obtained if excision had occurred. A band of 801 bp was detected (Figure 4.10 and appendix 9) in individuals only expressing the Hr5*ie1*-ZsGreen marker, consistent with the loss of the *Opie2*-DsRed marker and ampicillin resistance gene. Several controls were included in the PCR, such as presence/absence of the *Opie2* promoter sequence, *ie1* enhancer sequence, AmpR sequence, *attL* and *attR* junctions. In addition, PCRs were also performed on OX4580[4540A] parental strain gDNA. Based on the excision product (Figure 4.6), the *Opie2*-DsRed marker, the AmpR and the attR sequences should have been excised by the recombinase and therefore should not be present in the excised gDNA, but should be present in the parental gDNA. The *ie1* enhancer and *attL* sequences were not flanked by the recombination sites and should be present in both gDNAs.

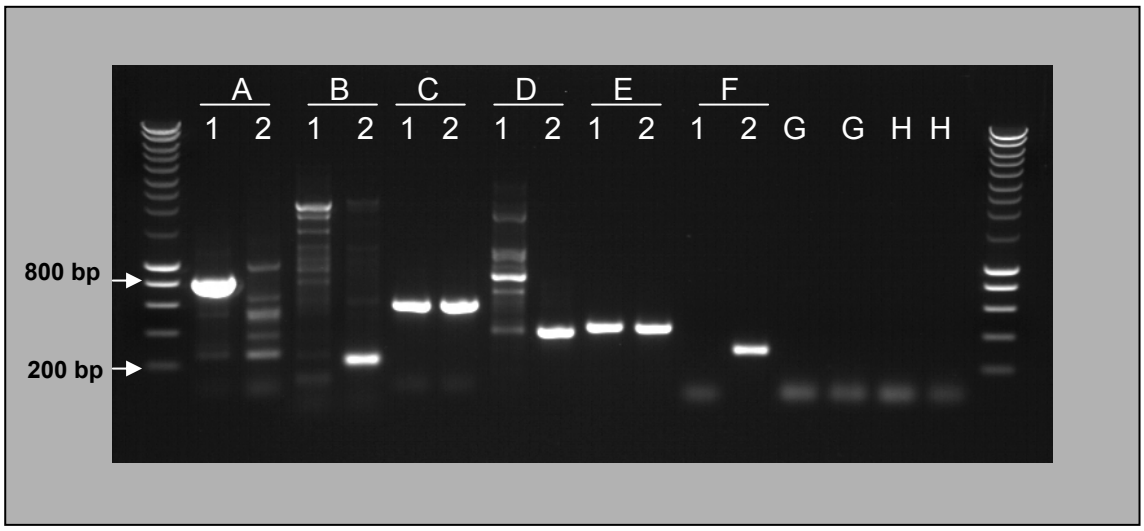

Figure 4.10. PCR evidence of Cre-mediated site-specific germline excision. Numbers 1 and 2 indicate PCR was done on gDNA from excised individuals and from parental OX4580[OX4540] line individuals, respectively. The letters A-H represent product bands for: A, excision junction (801 bp); B, Opie (202 bp); C, *ie1* (555 bp); D, AmpR (250 bp); E, attL (425 bp); F, attR (380 bp); G, wild-type control (801 bp) excision junction primers; and H, no gDNA control (801 bp) excision junction primers. Primer sequences can be found in appendix 3.

Furthermore, the putative excision band was cloned and sequenced to confirm the excision event (Table 4.7). The recombination event leading to excision was precise and no loss or addition of any DNA, other than predicted, occurred.

Table 4.7. Sequence analysis of the excision junction. *FRT* and *loxP* sequences are in blue and purple, respectively, and are flanked by OX4540, in red, and OX4580, in green. Primer sequences used for amplification are underlined.

CATGATGTGTGACAGTGGTACGAAGTATATGATAAATGGAATGCCTTATTTGGGAAGAGGAA
CACAGACCAACGGAGTACCACTCGGTGAATACTACGTGAAGGAGTTATCAAAGCCTGTGCAC
GGTAGTTGTCGTAATATTACGTGTGACAATTGGTTCACCTCAATCCCTTTGGCAAAAAACTT
ACTACAAGAACCGTATAAGTTCGAGATCGGCCGGCCTAGCTCGAAGAAGTTCCTATTCCGAA
GTTCCTATTCTCTAGAAAGTATAGGAACTTCCCTAGGATAACTTCGTATAATGTATGCTATA
CGAAGTTATCCTGCGGCGCGCCTCGCGTTAAGATACATTGATGAGTTTGGACAAACCACAAC
TAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAA
CCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTT
CAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGA
TTATGATCAGTTATCTAGATCCGGTGGATCTTACGGGTCCTCCACCTTCCGCTTTTTCTTGG
GTCGAGATCTGAGTCCGGAGGGCAAGGCGGAGCCGGAGGCGATGGCGTGCTCGGTCAGGTGC
CACTTCTGGTTCTTGGCGTCGCTGCGGTCCTCGCGGGTCAGCTTGTGCTGGATGAAGTGCCA
GTCGGGCATCTTGCGGGGCACGGACTTGGCCTTGTACACGGTGTCGAACTGGCAGCG

4.3.3.4. FLP-induced germline excision

The Flp-induced excision assay was performed in the same fashion as the Cre-induced excision assay. A total of 1395 OX4580[4540A] embryos were microinjected with Flp recombinase capped mRNA. Out of these, 201 (14.4%) survived to pupation. Phenotype analysis showed that, out of the 201, 55 were wild-type, 129 showed different *Opie2*-DsRed marker intensity and patchy expression patterns, and 17 individuals did not show *Opie2*-DsRed expression, only Hr5*ie1*-ZsGreen marker expression. All transgenic progeny were crossed to wild-type mates, individually or in pools of 2-5 transgenics. Out of 65 crosses, two did not produce any progeny; one did not generate any progeny expressing only the Hr5ie1-ZsGreen marker, and all of the remaining 63 crosses generated progeny expressing only the Hr5*ie1*-ZsGreen marker (Figure 4.11). Excision efficiency within a cross varied from 0.47% to 100% (raw data in appendix 8).

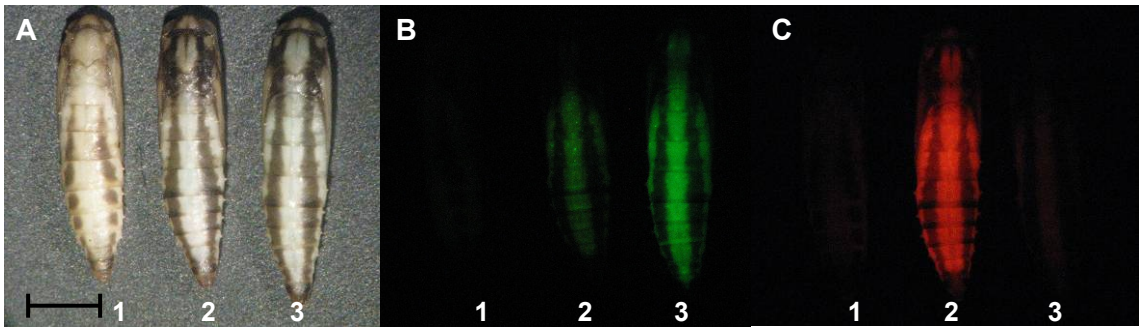

Figure 4.11. Flp-mediated excision fluorescent phenotype (pupae, dorsal view). (A) white light; (B) green excitation light and filters; and (C) red excitation light and filters. Numbers indicate (1) wild-type pupa; (2) parental OX4580[OX4540A] pupa and (3) pupa expressing the post-excision phenotype. Scale bar represents 2 mm.

As for Cre-induced excision, the putative excision event was confirmed by PCR, using the same primers. PCR analysis showed that individuals expressing only the Hr5*ie1*-ZsGreen marker produced a 801-bp fragment, consistent with the loss of the *Opie2*-DsRed marker and ampicillin resistance gene (Figure 4.12 and appendix 9). As

for the Cre-mediated excision, several controls were included in the PCR. The *Opie2* promoter, the AmpR and the *attR* sequence should have been excised by the recombinase and therefore should not be present in the excised gDNA, but should be present in the parental gDNA. The *ie1* enhancer and *attL* sequences were not flanked by the recombination sites and should be present in both gDNAs.

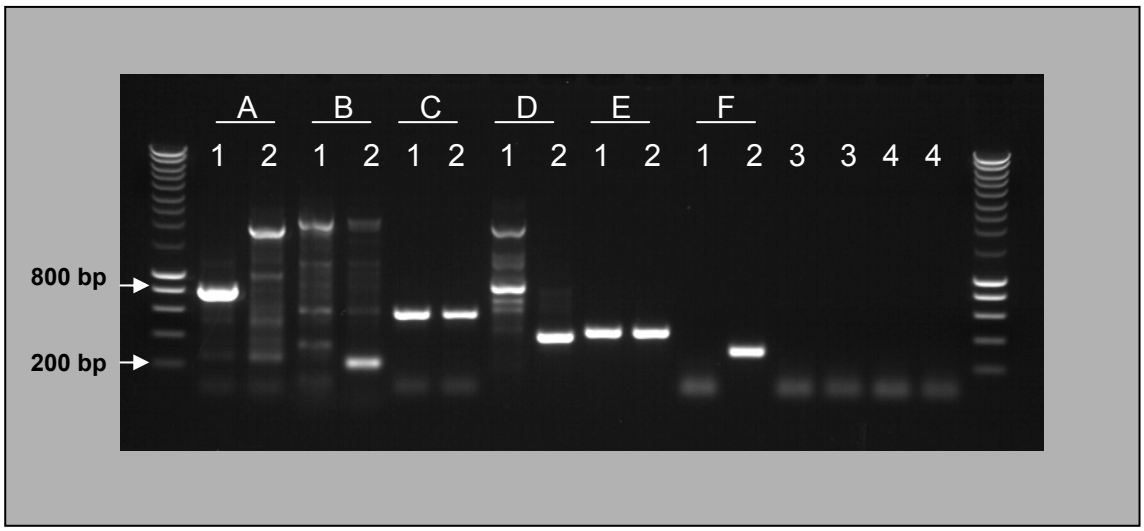

Figure 4.12. PCR evidence of Flp-mediated site-specific germline excision. Numbers 1 and 2 indicate PCR was done on gDNA from excised individuals and from parental OX4580[OX4540] line individuals, respectively. The letters A-H represent product bands for: A, excision junction (801 bp); B, *Opie2* (202 bp); C, *ie1* (555 bp); D, AmpR (250 bp); E, *attL* (425 bp); F, *attR* (380 bp); G, wild-type control (excision junction); and H, no gDNA control (excision junction). Primer sequences can be found in appendix 3.

The putative excision amplification product was cloned and sequenced confirming the expected excision junction, the flanking sequences (data not shown, as it is identical to the Cre excision event due to the same orientation and adjacent position of the two sites at both sides of the to be excised DNA segment).

4.3.4. Recombinase-mediated cassette exchange

In order to further improve the integration of transgenes into specific genomic locations, by inserting cassettes without plasmid backbone, recombinase-mediated cassette exchange (RMCE) was attempted in diamondback moth. RMCE integrates only the sequences between two recombination sites, avoiding the integration of the rest of the plasmid and its undesirable sequences, unlike the ΦC31-integrase system. Furthermore, in RMCE, cassettes would be inserted in one single cassette exchange step, instead of the two step integration-recombination system described above.

To test the potential use of RMCE, two different strategies were put in place. Firstly, I have attempted RMCE by flanking transgenes with one *loxP* and one *FRT* site and using both Cre and Flp recombinases simultaneously. Second, I have attempted RMCE by flanking transgenes with heterotypic *loxP* and *FRT* recombination sites.

4.3.4.1. RMCE using homotypic *loxP* and *FRT* sites simoultaniously

This strategy, proposed and demonstrated in mouse embryonic stem cells by Lauth and colleagues (2002), combines the Cre and Flp recombination pathways to achieve stable RMCE. This method is based on the Cre-*loxP* system in the 5' and the Flp-*FRT* system in the 3' end of the cassette of interest (Figure 4.13). As the *loxP* and *FRT* sites have no sequence similarity, there should not be cross-recombination between sites, and the cassette should be inserted into the genomic docking site with the same sites.

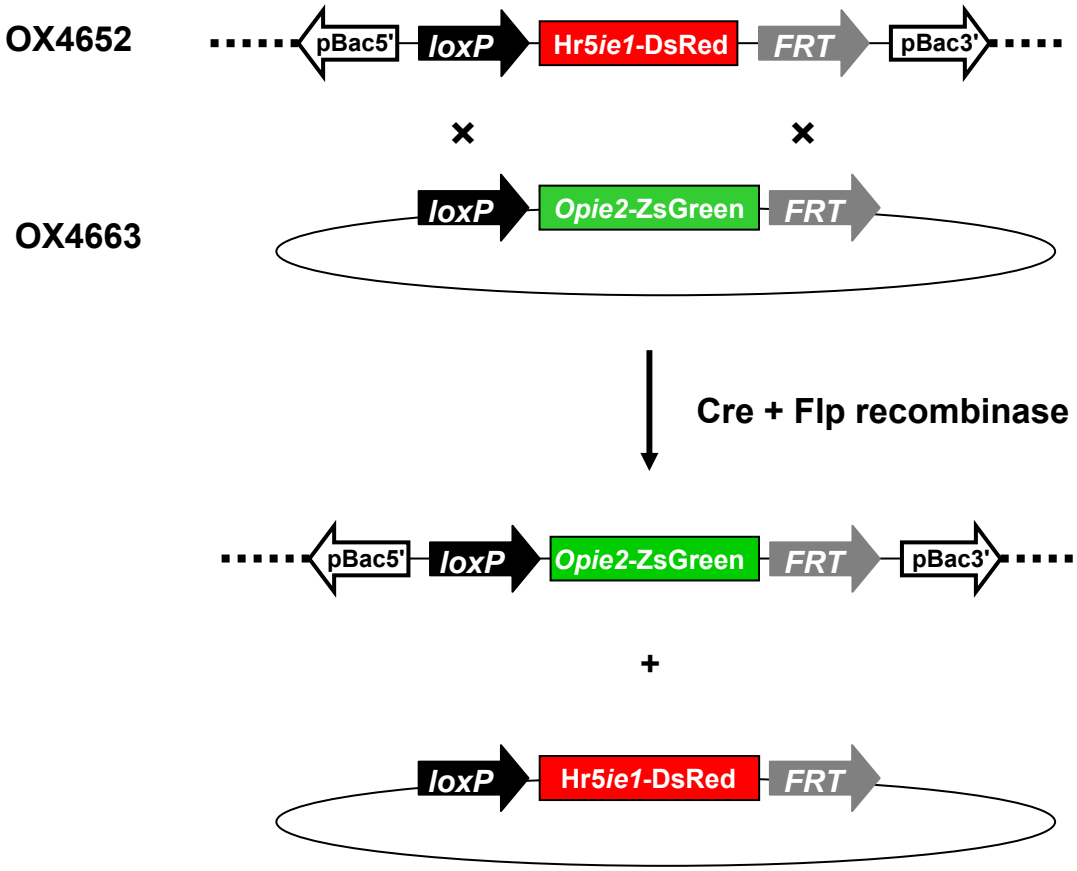

Figure 4.13. Schematic overview of the RMCE using one loxP and one FRT site flanking the cassette to be exchanged.

The recipient cassette, OX4652, was constructed in a *piggyBac* vector by flanking a Hr5*ie1*-DsRed2-nls-SV40 with a *loxP* and a *FRT* site, directly orientated (Figure 4.13). This target was introduced into wild-type diamondback moth using standard *piggyBac*-mediated transformation. A total of 1531 wild-type embryos were injected. Out of these, 396 (25.9%) matured to pupation and were crossed to wild-type mates in pools, of 2-5 injection survivors. Progeny were screened for Hr5*ie1*-DsRed2 expression and a total of five independent lines were established (OX4652A-E) (Figure 4.14).

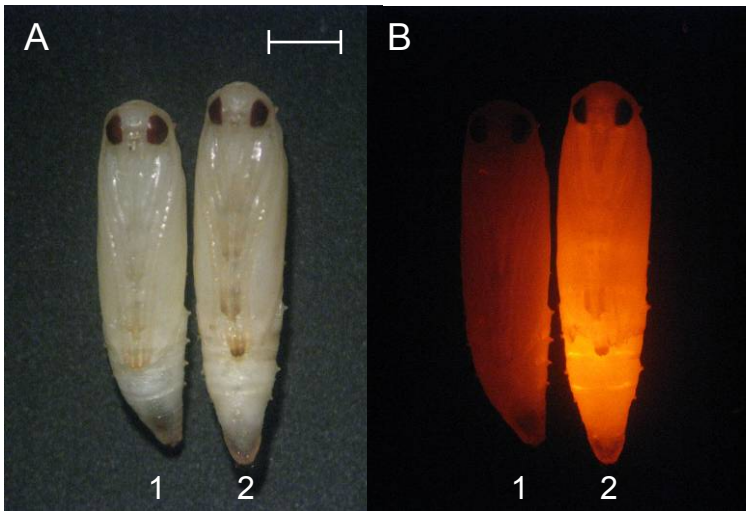

Figure 4.14. Flp-mediated RMCE fluorescent phenotype (pupae, frontal view). (A) white light; (B) red excitation light and filters. Numbers indicate (1) wild-type pupa; (2) OX4652B pupa. Scale bar represents 2 mm.

A donor plasmid, OX4663, was created by flanking an *Opie2*-ZsGreen-nls-Sv40 cassette with a *loxP* and a *FRT* site, directly orientated (Figure 4.13). OX4663 was co-injected with a 700 ng/µl mixture of Cre and Flp recombinase capped mRNA into lines OX4652B and OX4652D (Table 4.8).

Table 4.8. RMCE injection data, using one l*oxP* and one *FRT* site.

Line	No. embryos injected	Total no. injection survivors (G0)		No. integration events	Efficiency (%)
		Transgenic Go´s	Wild-type Go´s		
OX4652B	1246	177	78	0	-
OX4652D	862	184	66	0	-

For line OX4652B, a total of 255 (20.5%) out of 1246 injected embryos survived to pupation. Transgenic survivors carrying the recipient line marker gene, Hr5*ie1*-DsRed2, were crossed to wild-type mates in pools of 5-10 transgenics. Progeny were

screened for ZsGreen. If cassette exchange had occurred in the germline, progeny should express the donor plasmid *Opie2*-ZsGreen marker instead of the recipient line Hr5*ie1*-DsRed2 marker, as the plasmid carrying this marker should be lost during cell division. For line OX4652D, a total 862 were injected and 250 (29.0%) survived to pupation. Wild-type survivors were discarded and the remaining crossed to wild-type in pools of 5 transgenics. Progeny were screened for *Opie2*-ZsGreen marker expression, as for OX4652B. Individuals expressing the *Opie2*-ZsGreen marker were not detected in either line.

4.3.4.2. RMCE using heterospecific sites

Several functional *loxP* and *FRT* heterospecific sites have been developed by mutating the wild-type site spacer region (Lee & Saito, 1998; Bode et al., 2000; Turan et al., 2010). Among these, the *lox2272* and the *FRT3* (Figure 4.15) have been shown to be particularly effective at recombining to another identical sequence, but not recombining to the wild-type sequence (Lee & Saito, 1998; Kolb, 2001; Langer et al., 2002; Bode et al., 2000; Turan et al., 2010). RMCE using these sites has also been demonstrated by Oberstein and colleagues (2005), and Horn and Handler (2005), in *D. melanogaster*. Therefore, these two sites were chosen for this work, together with their wild-type counterparts. Although, symmetric element ´c´ from the FRT DNA sequence is not required for Flp-mediated recombination (Proteau et al., 1986), a full FRT DNA sequence was used throughout this study.

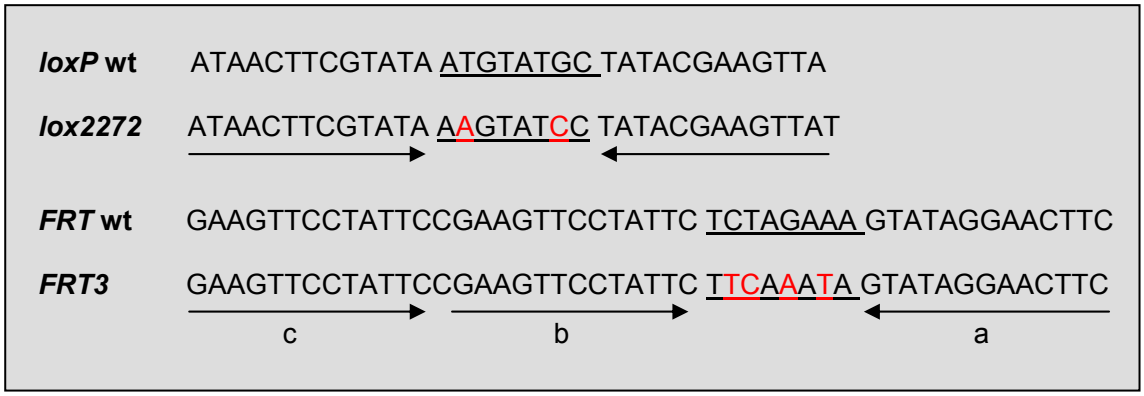

Figure 4.15. Wild-type (wt) and mutant *lox2272* and *FRT3* sequences. Horizontal arrows denote the 13-bp symmetric elements surrounding the 8-bp spacer region (underlined). The FRT site is composed of three symmetric elements termed ´a´, ´b´ and ´c´ from right to left. Mutations in the spacer region are shown in red.

A recipient *piggyBac* construct, OX4655, harbouring a Hr5*ie1*-DsRed marker gene flanked by a FRT and a loxP site at the 5' end, and a loxP2272 and a FRT3 site on the 3' end, was built and integrated into the genome by standard *piggyBac* transformation (Figure 4.16).

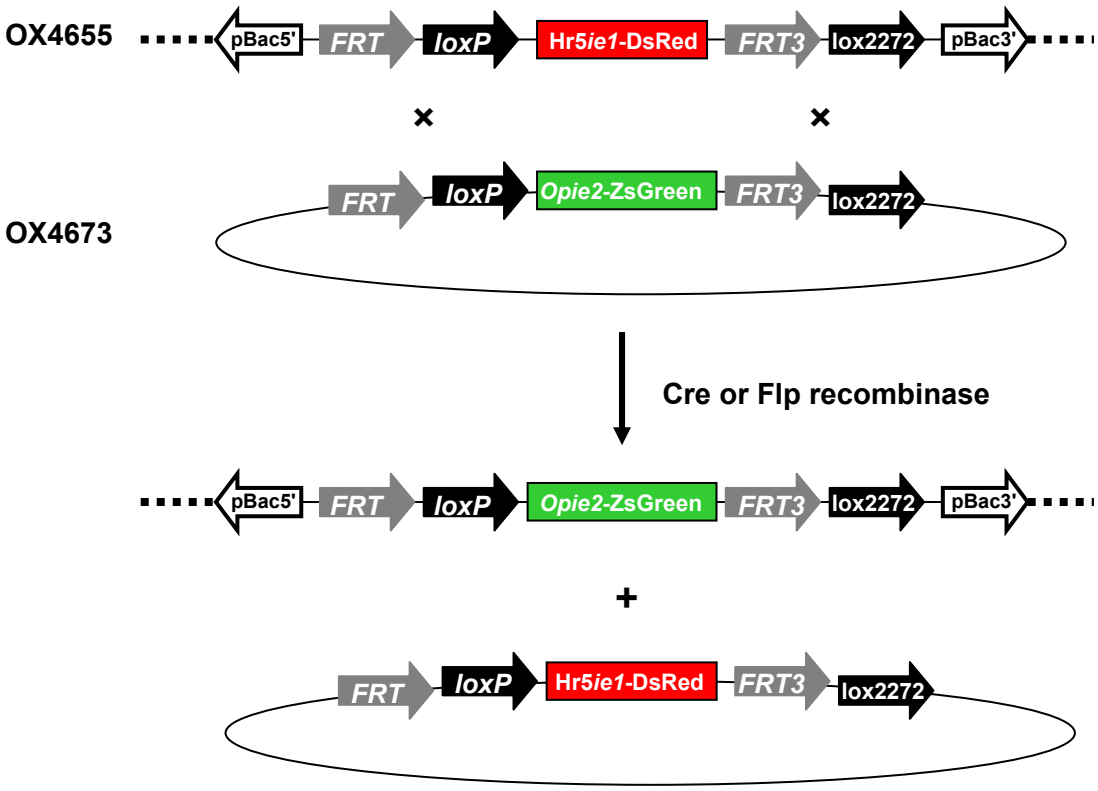

Figure 4.16. Schematic overview of RMCE using *loxP* and *FRT* heterospecific sites.

A total of 1530 wild-type embryos were injected with OX4655. Out of these, 687 (44.9%) survived to pupation and were crossed to wild-type mates, in pools of 2-5 transgenics. Progeny were screened for DsRed expression and six independent transgenic lines were established (OX4655A-F) (Figure 4.17)

Figure 4.17. Flp-mediated excision fluorescent phenotype (pupae, dorsal view). (A) white light; (B) red excitation light and filters. Numbers indicate (1) OX4655 pupa; (2) wild-type pupa. Scale bar represents 2 mm.

A donor plasmid, OX4673 consisting of a *Opie2*-ZsGreen cassette flanked, at the same ends, by the same wild-type and mutant *loxP* and *FRT* sites as OX4655, was built and co-injected into the recipient OX4655D line (Table 4.9) with either Cre or Flp recombinase capped mRNA. The recipient OX4655D line consisted only of hemizygous individuals and was, therefore, enriched for the transgene but not fully homozygous.

Table. 4.9. RMCE injection data, using l*oxP* and *FRT* heterospecific sites.					
Recombinase	**No. Embryos injected**	**Total no. injection survivors (G0)**		**No. Integration events**	**Efficiency (%)**
		Transgenic Go´s	**Wild-type Go´s**		
Cre	1141	110	45	0	-
Flp	1466	122	38	0	-

Wild-type progeny was discarded and progeny expressing the Hr5*ie1*-DsRed marker were crossed to wild-type mates in pools of 5 transgenics. No RMCE events were detected in the OX4655D line, using Cre or Flp recombinase.

4.4. Discussion

This chapter presents the first site-specific genome manipulation in diamondback moth, and in Lepidoptera generally, using the ΦC31 integrase, and the Cre- and Flp-recombinases.

ΦC31 integrase mediates cassette integration in the diamondback moth germline

To provide an alternative to the random transgene insertion caused by transposable elements, targeted integration of a transgene was successfully achieved using the ΦC31 integrase. Out of three OX4389 recipient lines, two lines were transformed by site-specific integration using a donor plasmid, carrying an *attB* site, co-injected with ΦC31 integrase mRNA. Successful ΦC31-mediated integration was also achieved using the OX4540 recipient line and the OX4580 donor plasmid.

The observation that some but not all recipient lines resulted in integration may indicate that integration efficiency depends on the random genomic location of the recipient construct, as previously observed in *Drosophila* (Bischof et al., 2007) and in mouse embryonic stem cells (Monetti et al., 2011). In some locations, surrounding chromatin configuration and/or repetitive sequences might prevent the integrase from locating, and/or attaching to its cognate recognition sites. Some DNA sequences surrounding the *attP* site also seem to interfere with the recombination reaction. This was indicated when injection of the donor plasmid OX4034 into OX3441A, C and H recipient lines consistently failed to produce site-specific transformants. Previous experiments in pink bollworm, using the same OX3441 recipient construct, also failed to produce integration events. This was true even with the donor plasmid OX4105 that successfully resulted in ΦC31-mediated integration in mosquitoes (Labbé et al., 2010)

was used.. In the OX3441 recipient construct, the *attP* site is approximately 2.5× closer to the neighbouring promoter than in the OX4389 and OX4540 recipient constructs. Furthermore, in OX3441 the marker promoter is Hr5*ie1*, a highly repetitive sequence. In OX4389 and OX4540 the regulatory sequence is *Opie2*, with no repetitive elements. Therefore, secondary structure and repetitive elements surrounding the *attP* site do seem to have an adverse influence on the recombination reaction.

The estimated integration efficiency was between 0.16% and 0.39%, depending on the recipient line. Differences in efficiency could be due to the *attP* genomic location, with the transgene in line B sited in a more favourable location for recombination to occur. Estimated integration efficiencies were consistently lower than those estimated in *Ae. aegypti* (Nimmo et al., 2006) and *Ae. albopictus* (Labbé et al., 2010), 23% and 1.24%, respectively. Using the ΦC31 system in *Ae. aegypti*, Nimmo and colleagues (2006) recorded an integration efficiency up to 7.9-fold higher than with *piggyBac*. In diamondback moth, though, ΦC31-mediated integration efficiency was lower, between 0.16 and 0.39%. Higher estimated transformation efficiencies of between 0.48% and 0.68%, have been routinely achieved with *piggyBac* in the course of this work (see Chapter 3). Nevertheless, given that, for each transgene inserted using *piggyBac*, at least several lines would typically have to be generated and analysed and how laborious this can be, such efficiencies are still advantageous for genomic engineering practices. After the initial identification and characterisation of a suitable docking strain, there would be no need to generate further insertions, thereby avoiding all the laborious work associated with their production and analysis.

Combined used of ΦC31 integrase and Cre/Flp recombinases allows site-specific integration of transgenes followed by excision of unwanted plasmid sequences

The ΦC31 integrase was shown to mediate site-specific integration in diamondback moth. However, one limitation of this system is the integration of the entire donor plasmid. To take advantage of the ΦC31 system for the stable integration of transgenes with subsequent excision of the donor plasmid backbone, the ΦC31 system was combined with the Cre and Flp system.

Following ΦC31-mediated integration of the OX4580 donor plasmid into OX4540A recipient line, the *loxP* and *FRT* sites in each donor and recipient construct flanked the donor plasmid backbone and the marker gene of the recipient construct. Post-integration injection of Cre or Flp recombinase mRNA, mediated recombination of their respective cognate sites, resulting in the excision of the DNA sequences between them. As a result, unwanted plasmid backbone integrated, including its antibiotic resistance gene (ampicillin), was successfully removed from the strain. Excision mediated by both Cre and Flp recombinases occurred at high efficiency, in 90.4% and 100% of fertile families, respectively. Cre-mediated excision frequencies ranged between 0.88% and 100%, and Flp-mediated excision frequencies ranged between 0.47% and 100% within families. These results are comparable to those obtained in *Drosophila* and *Ae. aegypti* (Table 4.10).

Table 4.10. Excision frequencies for Cre and Flp recombinases in other insects		
Author	Excision efficiency	
	Cre recombinase	Flp recombinase
Golic & Lindquist (1989)	N/A	49.4 – 96.4% (*D. melanogaster*)
Siegal & Hartl (1996)	0 – 100% (*D. melanogaster*)	N/A
Jesinskiene *et al.* (2003)	20 – 99.4% (*Ae. aegypti*)	0% (*Ae. aegypti*)

This three-step approach allows the integration of any sequence of interest in a suitable genomic location using the ΦC31 system, leaving Cre or Flp recombinase available for downstream excision of unwanted DNA sequences. As a result, limitations imposed by position effects are overcome. In addition, plasmid backbone and antibiotic resistance sequences, which are an impediment to the release of transgenic insects, are removed from the insect genome.

Recombinase-mediated cassette exchange

An improvement to the above approach would be use of RMCE-based DNA integration which allows the insertion of target cassettes containing just the desired sequences, excluding the vector backbone, into pre-engineered docking sites in one step. Three different strategies were adopted to try to attain RMCE: one strategy combined the Cre/*lox* and Flp/*FRT* systems, one a modified Cre/*lox* and the other a modified Flp/*FRT* system. None of these systems gave germ-line transformants. RMCE combining both Cre/*lox* and Flp/*FRT* systems has been successfully reported in mouse embryonic stem cells (Lauth et al., 2002) but has not been described in insects. One difference between the work reported here and that of Lauth et al., was

that they used a DNA helper plasmid carrying both Cre and Flp recombinases coding sequences, whereas in this case mRNA of both recombinases were co-injected together. Injection mixes usually contain up to 1000 ng of DNA or DNA and mRNA mix. Concentrations above 1000ng make the mix too viscous and difficult to inject. In order to make an injection mix composed of donor plasmid and Cre and Flp mRNA without exceeding the 1000 ng limit, only 350 ng of each mRNA was used, compared to the 700 ng used in the excision assay. Lower concentrations of mRNA injected and relatively fast mRNA degradation might have resulted in too little enzymes available for recombination. In general, lower concentrations of DNA helper plasmid are used in injection mixes, therefore the use of a DNA helper plasmid should be considered in future work. Injection of a higher number of embryos should also be considered. If the efficiency of recombination is quiet low, more embryos need to be injected to increase the probability of one recombination event occurring.

RMCE using *loxP* and *lox2272* and *FRT* and *FRT3* sites has been achieved in *Drosophila melanogaster* (Horn & Handler, 2005; Oberstein et al., 2005). Therefore, and taking into account both recombinases were shown to be active in diamondback moth, it was expected that both systems would be transferable to diamondback moth. The efficiency of cassette exchange has been shown to be dependent on the DNA surrounding the recombination sites (Nakano et al., 2001; Mlynárová et al., 2002). The presence of palindromic non-*lox* DNA interferes with the homology search of the Cre recombinase adversely affecting recombination (Mlynárová et al., 2002). As only one recipient line was injected in this experiment, it is impossible to determine if failure was due to the genomic location of the recipient construct or to other factors. To fully test the potential of RMCE using *lox* or *FRT* heterospecifc sites, more

recipient lines would have to be injected and analysed. Although more recipient lines were available, due to time limitations, it was not possible to test more lines.

Chapter 5

Promoters for the development of conditional female sterility in pink bollworm

5.1. Introduction

Engineered female sterility to improve conventional SIT

Conventional SIT uses radiation to induce chromosomal damage in the gamets or their precursors, rendering the insects sterile (previously discussed in Chapter 1, section 1.3). However, radiation also damages somatic cells. This decreases the competitiveness of the sterile insects, especially in Lepidoptera, where high doses of radiation are needed to achieve sterilisation (Seth & Sharma, 2001). Control strategies based on the use of transgenesis to induce sterility would eliminate the need for sterilisation by radiation. The released insects would be fitter and more competitive, unless the genetic engineering causes a greater reduction in performance than the radiation-sterilisation alternative. A genetically engineered female-sterile strain could potentially be a valuable tool in pink bollworm SIT control programmes, where bisex releases are common practice. Released females are considered to be an important element of the programme, as a major component of moth reproduction is the long-range attraction of males by female-produced sex pheromones (Allison & Cardé, 2005). In effect, they may add to the control effect by providing an element of mating disruption. In such a system, no progeny would be produced from mating between sterile females and wild-type males which would reduce the reproductive potential of the wild-type population. Neverthless, a female-sterile system would have to be combined with a male-sterily system, so both females and males to be released should be sterile, for maximum control effect. In such scenario, no progeny would be produced from mating between sterile moths and wild-type moths which, after several sterile female releases over a target area, the native wild-type population density would decrease.

Oogenesis and the development of a female sterile strain

One potential method of inducing female sterility might be the use of regulatory sequence (e.g. a promoter) from a gene involved in oogenesis. In insects such as *B. mori* and *D. melanogaster*, the follicle is the functional development unit in oogenesis. It consists of an oocyte, nurse cells and a layer of follicular cells. All three types of cells in a developing follicle are highly specialised with respect to their functions during oogenesis (Sauman & Berry 1993). The oocyte itself is transcriptionally inactive until the stage of midblastula transition after fertilisation. Therefore, all materials necessary for oocyte development must be maternally synthesised in cells other than the oocyte (Sauman & Berry 1993): the nurse cells synthesise maternal RNA and facilitate its transport to the oocyte, the fat body synthesises several proteins, such as vitellogenins, and the follicle cells synthesise chorion proteins and some vitellogenins, and facilitate the uptake of various materials from the haemolymph to the oocyte.

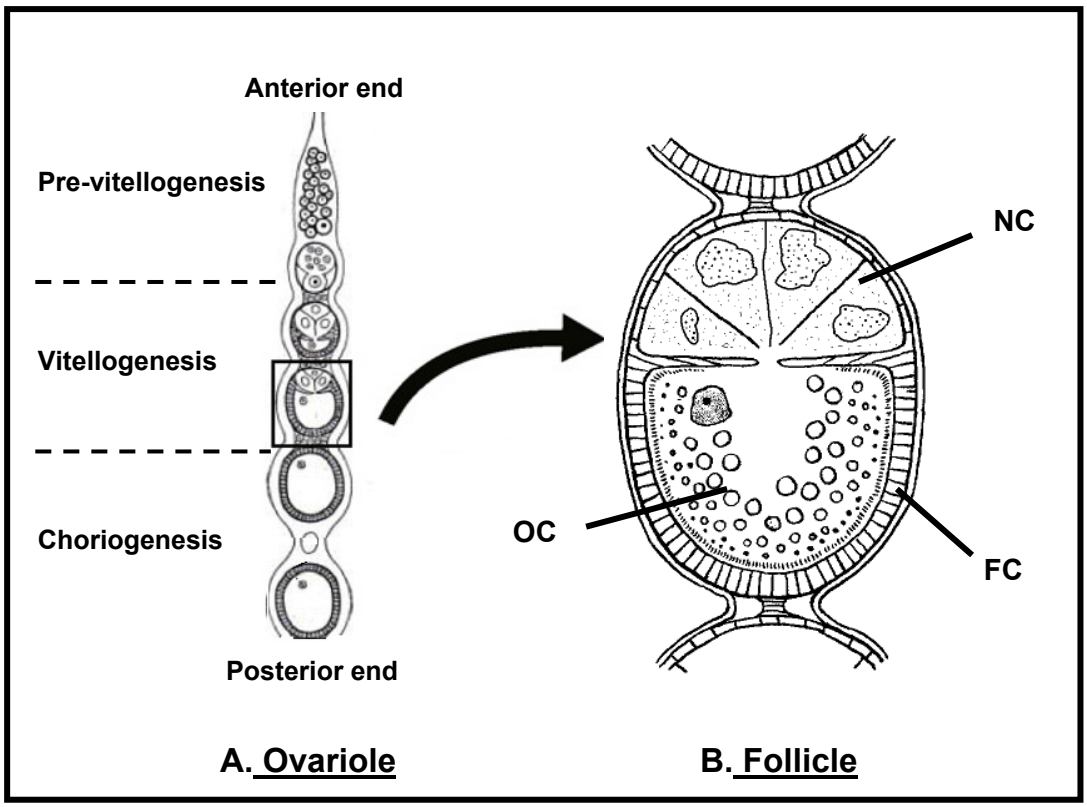

Figure 5.1. Diagram of a *B. mori* ovariole and follicle as seen in median longitudinal section. A. The ovary consists of four ovarioles. Each ovariole consists of an array of follicles at different stages of development. Follicles are formed in the anterior end and mature as they move towards the posterior end, passing through three stages of development: pre-vitellogenesis, vitellogenesis and choriogenesis. B. In the follicle, the oocyte (OC) is connected to the nurse cells (NC) through an intercellular bridge. Surrounding the oocyte and the nurse cells is a layer of follicle cells (FC) (Adapted from Sonobe and Ito, 2009)

Promoters of maternally expressed genes produced in the nurse and/or follicle cells could be used to drive the expression of a lethal effector gene, in a tissue- and developmental stage-specific manner, in those cells. This would be expected to adversely affect oogenesis, hamper oocyte development and, consequently, render the females sterile. However, up until the present, genes involved in pink bollworm oogenesis have not been characterised. An alternative would be to test promoters of characterised genes involved in *B. mori* oogenesis in pink bollworm. *B. mori* gene promoters have been successfully used in other lepidopteran species. The *B. mori* A3 cytoplasmic actin gene (*Bm*A3) has been used to express fluorescent proteins in transgenic pink bollworm (Peloquin et al., 2000; Simmons et al., 2011) and the

codling moth (Ferguson et al., 2010). Gene promoters from a more phylogenetically distant species, *D. melanogaster*, have also been used in the transformation of the codling moth (Ferguson et al., 2010) and were shown to be transiently active in embryos of the potato tuber moth, *Phthorimaea operculella* (Mohammed and Coates, 2002). If *B. mori* ovary-specific gene promoters were shown to be active in the pink bollworm, they could potentially be used to render pink bollworm females sterile, as described above.

Conditional female sterility: the tetracycline-repressible system

For mass rearing purposes, sterility would have to be conditional. Expression of the transgene has to be repressed under permissive rearing conditions in the laboratory so the strain can be reared. Just before or after release, a change to non-repressed conditions will result in sterility. Conditional sterility could be achieved by controlling the expression of the effector gene using the two-component tetracycline-repressible system described in Chapter 1. Experimental development and testing of such a system could in principle be efficiently achieved by generating and crossing together two different insertions or strains: a driver strain carrying of the tetracycline-repressible transactivator (tTA) driven by an ovary-specific gene promoter, and an effector strain carrying a lethal effector gene under the transcriptional control of the tTA-responsive *tetO* sequence, to which the tTA binds, leading to transcription of the effector gene. This allows the two components to be developed and tested independently, and greatly reduces – compared with the use of constructs that carry both system components on the same construct – the risk of failure due to misregulation of effector expression leading to an inability to generate transgenics. After obtaining transgenic lines, the two strains would be crossed to each other.

Progeny carrying both transgenes should be fertile in the presence of tetracycline provided as a diet supplement in the laboratory rearing conditions. In the presence of tetracycline, tTA will bind to tetracycline instead of binding to *tetO*, ensuring that the lethal gene is not expressed. In the absence of tetracycline, tTA, expressed in the ovary, should bind to *tetO* and induce expression of the lethal effector gene, rendering the females sterile, or at least with a marked reduction in fertility.

Aim

In this chapter, different putative promoters of genes involved in *B. mori* oogenesis were tested in pink bollworm, in order to determine their activity in pink bollworm and their potential use in the development of a conditional female-sterile pink bollworm strain. Candidate putative promoters, shown to be active in pink bollworm, were then used to try to attain conditional female sterility using the tetracycline-repressible system. Female-specific expression of the *D. melanogaster* pro-apoptotic gene *reaper*^{KR} could, in principle, cause female sterility when driven by the tetracycline-controlled transactivator (tTA) under the regulation of an ovary-specific gene promoter.

5.2 Materials and Methods

5.2.1. Generation of DNA constructs

5.2.1.1. OX4360

To build this construct, the previously identified putative chorion peroxidase promoter and 5' UTR fragment was amplified by PCR from construct OX4270 pB[Opie-DsRed-nls-SV403'UTR-BmChxtpro-ZsGreen-nls-SV403'UTR], in two steps. A 1.8 kb putative promoter fragment was amplified using Platinum *Taq* High Fidelity Polymerase with primers '928' and '944' (see appendix 3), containing *Sac*I and *Bam*HI restriction sites at their ends, respectively. A second 2.7 kb putative chorion peroxidase promoter fragment was amplified by AccuPrime^{TM} *Pfx* DNA Polymerase with primers '927' and '945' (see appendix 3). The two putative promoter fragments were ligated together and used to replace the *D. melanogaster* 316 promoter fragment of OX4301, pB[Dm316pro-tTAV-Hr5*ie1*-ZsGreen-nls-SV403'UTR] which was removed using *Sac*I/*Bam*HI , creating OX4360, pB[BmChPxtpro-tTAV-Hr5*ie1*-ZsGreen-nls-SV403'UTR].

5.2.1.2. OX4413

Construct OX4433, psc[*attB*-Hr5ie1-nls-DsRed2-nls-SV403'UTR] was made by digesting construct OX4259, psc[*attB*-hr5*ie1*-ZsGreen-SV403'UTR], sequentially, with *Bgl*II/*Age*I to remove the ZsGreen fragment, which was replaced with a nls-DsRed2-nls fragment. The nls-DsRed2-nls fragment was removed from construct OX4204, pB[Aetopi-nls-DsRed2-nls-3×P3-DsRed-SV403'UTR], by sequential digestion with *Bgl*II and *Age*I. The *Avr*II/*Xho*I *B. mori* egg-epecific protein putative promoter (BmESPpro) and 5' UTR (2551 bp) was amplified from *B. mori* gDNA

(Green strain) with *Pfx50*™ DNA Polymerase and primers 1004 and 1005. It was then inserted into OX4143, pB[Opie2-DsRed-nls-Sv403'UTR-BmAcpro-ZsGreen-nls-SV403'UTR], after the *B. mori* muscle Actin protein (BmAcpro) promoter sequence was removed with *Avr*II/*Xho*I, making OX4413, pB[Opie2-nls-DsRed-nls-Sv403'UTR-BmESPpro-ZsGreen-nls-SV403'UTR]. 5′-RACE was performed with the gene specific primer BmespRace and the nested primer BmespRacen, as described in Chapter 2, in order to verify for the presence of introns in the 5'UTR.

5.2.1.3. OX4498

OX4498 was made by replacing the *B. mori* chorion peroxidase putative promoter from OX4360 with the *B. mori* vitelline membrane protein VMP30 (BmVMP30) putative promoter and 5' UTR. The *B. mori* VMP30 putative promoter (2099bp) was amplified from *B. mori* (Green strain) gDNA with *Pfx50*™ DNA Polymerase with primers BMVMP30AvrF and BMVMP30BamR, containing restriction sites for *Avr*II and *Bam*I, respectively. The *B. mori* chorion peroxidase putative promoter was removed with *Avr*II/*Bam*I and replaced with the *Avr*II/*Bam*HI *B. mori* VMP30 putative promoter fragment in the OX4360 construct to create OX4498, pB[BmVMP30pro-tTAV-Hr5*ie1*-nls-ZsGreen-nls-SV403'UTR]. 5′-RACE was performed with the gene specific primer BmVMP30GSP and the nested primer BmVMP30N, as described in Chapter 2, in order to verify for the presence of introns in the 5'UTR.

5.2.1.4. OX4521

The OX4521 construct was made by replacing the *B. mori* chorion peroxidase putative promoter from OX4360 with the *B. mori* egg-specific putative promoter from

OX4413. Both OX4360 and OX4413 were digested with *Avr*II/*Bam*HI and the *Avr*II/*Bam*HI BmChPxtpro removed from OX4360 and replaced with *Avr*II/*Bam*HI BmESPpro fragment, making OX4521, pB[BmESPpro-tTAV-Hr5*ie1*-ZsGreen-nls-SV403'UTR].

5.2.1.5. OX4660

OX4660 was made by replacing the *B. mori caudal* gene (BmCaudpro) putative promoter from OX4364, pB[Opie2-DsRed-nls-Sv403'UTR-BmCaudpro-ZsGreen-nls-SV403'UTR] with the *B. mori* VMP30 (BmVMP30) putative promoter. The BmVMP30 putative promoter was amplified from *B. mori* (Green strain) gDNA with *Pfx50*™ DNA Polymerase with primers BMVMp30AvrF and BMVMP30xhoR (see appendix 3), containing restriction sites for *Avr*II and *Xho*I, respectively. The BmCaudpro promoter was removed with *Avr*II/*Xho*I and replaced with the *Avr*II/*Xho*I putative promoter fragment in the OX4364 construct to create OX4660, pB[BmVMP30pro-ZsGreen-nls-Opie2-DsRed2-nls-SV403'UTR]. 5′-RACE was performed with the gene specific primer BmVMP30GSP and the nested primer BmVMP30N, as described in chapter 2, in order to verify for the presence of introns in the 5'UTR.

5.2.1.6. Other constructs

Construct OX4270 was made by Dr Sarah Scaife.

5.5.2. Dissection of pink bollworm females

Female genitalia were dissected under an Olympus SZX12 stereomicroscope. Adult moths were anaesthetised with CO_2 and ovaries were dissected from the female abdomens by gently pulling, with the help of a fine forceps, the very end of the abdomen. The ovaries were then placed in a vial with deionised water to avoid desiccation and to aid viewing. Fluorescent protein expression was detected using the same microscope with the appropriate filter set.

2.5.3. Egg production and egg hatch rate assay

Individuals of a tTAV-expressing strain (driver strain) and individuals of a *tetO*-lethal gene expressing strain (effector strain) were crossed together, in polls, in a ratio of 1:3 (♂:♀) (Figure 5.2). Eggs from this cross were collected every 2 days and divided into two groups: one group was reared under permissive conditions (provided with food without tetracycline), and one group was reared under repressive conditions (provided with food containing tetracycline). At pupal stage, the offspring were collected and screened for both strains' transformation markers. The numbers of pupae expressing both markers, only one of the markers or no marker was recorded. Ten females expressing both or only one marker were then individually crossed to wild-type males, reared off tetracycline, in 114 ml paper beakers (Ambicon UJK Ltd, London, UK) and reared as wild-type off tetracycline. Eggs produced by these crosses were collected every 2 days and its number recorded, throughout the moths' life span. Eggs were then placed in a Petri dish sealed with Parafilm and kept in the dark at 27± (1ºC) and 60% (±2%) RH until they hatched. The number of hatched eggs was recorded.

Crosses with females carrying both transgenes reared on-tetracycline, and crosses with wild-type females reared off tetracycline, were included as controls.

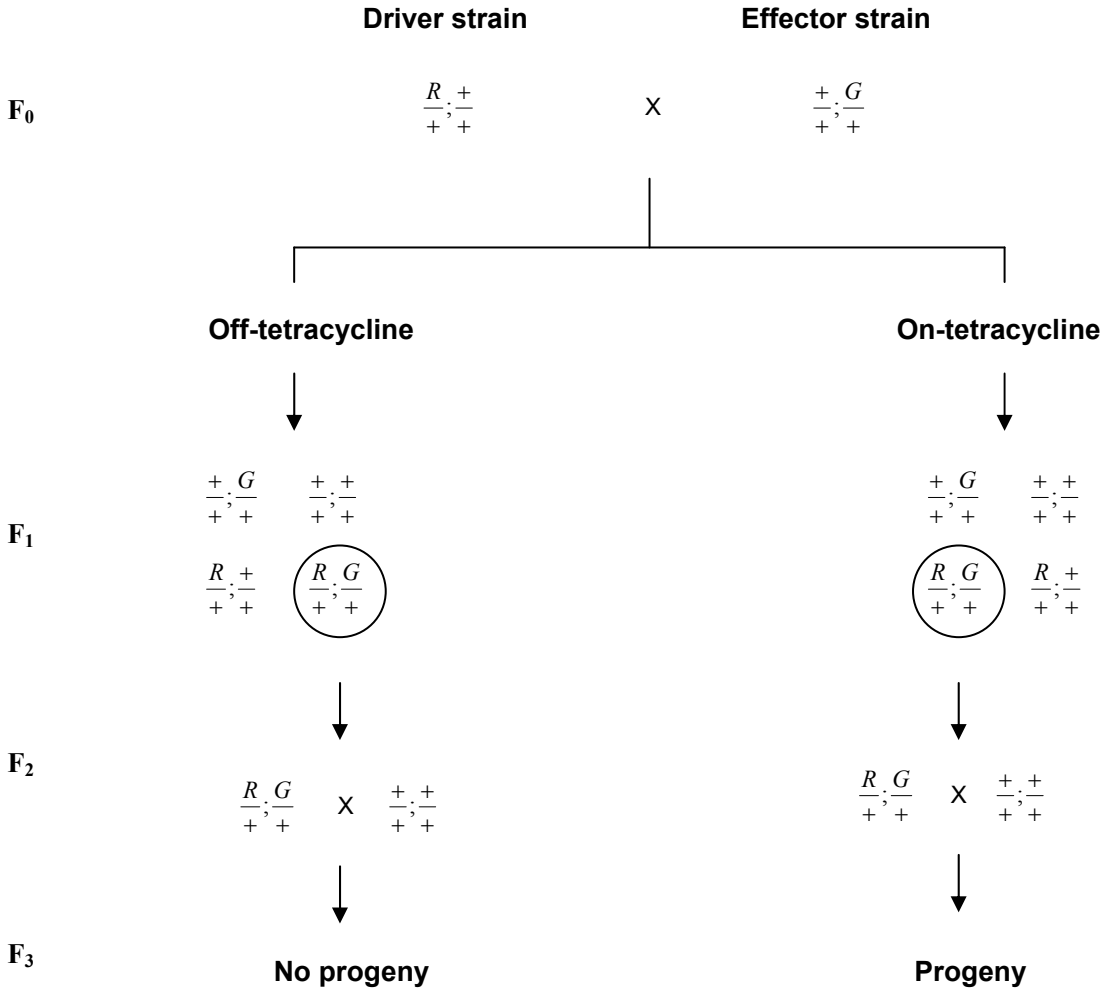

Figure 5.2. Scheme showing the genetic cross between hemizygous individuals of a driver and effector strain. 'R' represents the red fluorescent transformation marker of driver strain, and 'G' represents the green fluorescent transformation marker of the effector strain.

2.5.4. Statistical analysis

Data were analysed using IBM SPSS Statistics software (version 19.0; SPSS INC., Chicago, USA). Assumptions of normality were tested with Kolmogorov-Smirnov tests. Treatments were compared using one-way ANOVAs. When required, Dunnett's T3 *post hoc* test was used for pairwise comparisons, assuming unequal variances. In order to determine if differences from an observed and expected value were significant, the Chi-square teste (χ^2) was used.

5.3. Results

5.3.1. Candidate gene promoters

Little is known about pink bollworm genes due to lack of genomic data, which limits the use of pink bollworm ovary-specific gene promoters in this study. The silkworm *(B. mori*) is a model system for Lepidoptera, in molecular genetics and in structural and functional genomics. The genome of *B. mori* has been fully sequenced. This chapter describes the testing of sequences from silkworm genes in transgenic pink bollworm.

Based on a literature search, four candidate silkworm genes were selected to test in pink bollworm: ecdysteroid 22-kinase (EcKinase), vitelline membrane protein 30 (BmVMP30), egg-specific protein (ESP) and NTP1-4. These were the only ovary-specific genes found in the literature that have been previously characterised in *B. mori*.

The **EcKinase** catalyses the conversion of free ecdysteroids to physiologically inactive ecdysteroid 22-phosphates in the ovary-egg system, a process involved in the metabolism of the 20-hydroxyecdysone, the principal moulting hormone in insects, which plays an important role in early embryonic development (Itô & Sonobe, 2009; Yamada & Sonobe, 2003, 2004, 2006). EcKinase mRNA is produced in the nurse cells, during vitellogenesis, and is transferred to the oocyte where it is translated, and is also synthesised by the oocyte during choriogenesis (Sonobe & Itô, 2009). EcKinase orthologues are observed in almost all insect species whose complete genome sequences are available (Sonobe & Itô, 2009).

The *B. mori* chorion vitellin membrane protein 30 (**BmVMP30)** is involved in the maintenance of the structural integrity of the vitelline membrane and follicular

epithelium. It is synthesised by the follicle cells and then secreted onto the oocyte, forming part of the egg shell structure (vitelline membranes and chorion), during middle/late vitellogenesis (Kendirgi et al., 2002).

The egg-specific protein (**ESP**) is one of the three major yolk proteins in the *B. mori* yolk system (Kobayashi & Inagaki, 1989). In contrast to the other yolk proteins, which are synthesised in the fat body, the ESP is synthesised in the follicle cells of vitellogenic ovaries and sequestered into the developing oocyte (Irie & Yamashita, 1982; Sato & Yamashita. 1991; Inagaki & Yamashita, 1989).

The **NTP1-4** is synthesised in the follicle cells, exclusively at the end of vitellogenesis and beginning of choriogenesis. The function of this protein is unknown. However, judging by the tight developmental regulation of this ovary-specific transcript over the vitellogenic/choriogenic boundary, it might be involved in vitelline membrane and/or chorion formation (Glushek, 2001).

In addition to the above genes, the *B. mori* chorion peroxidase (**BmChPxd**) gene, previously cloned by a colleague, was also selected. In *D. melanogaster* and *Ae. aegypti* the peroxidase enzyme is involved in the formation of a rigid and insoluble chorion by catalysing chorion protein covalent cross-linking (Han et al., 2000; Margarits, 1985), thereby protecting the embryo from adverse environmental conditions during embryogenesis (Li et al., 1996).

5.3.2. Developmental profile of candidate gene transcripts

To confirm that the candidate genes were exclusively expressed in ovaries, the expression profile of the candidate genes transcripts were analysed by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RNA

was extracted from *B. mori* individuals, from all life stages, excluding pupae, and cDNAs were generated as described in Chapter 2 (section 2.15.3). Oligonucleotides were designed, whenever possible flanking an intron, to control the amplification of any possible contamination of genomic DNA in the sample. PCR product samples were amplified with 30, 35 and 40 thermal cycles.

RT-PCR analysis for the egg-specific protein, chorion vitellin membrane protein 30 and chorion peroxidase genes, indicated that transcripts were detected in female ovaries but not detectable in carcasses of female adults or any other developmental stage (Figure 5.3). The only exception was the NTP1-4, where transcripts were detected in third larval instar. Glushek (2001) used Northern Blot analysis to analyse the transcript expression profile in different adult tissues. These did not include other developmental stages besides adult female. Transcript expression in the third larval instar limits the use of this gene promoter in the development of a female sterile system. It would be difficult to rear the insect to adulthood in the generation prior to release: to induce ovary-specific effector gene expression, the insects would need to be reared off tetracycline. If the effector gene was also expressed in the third instar, the insects would likely be killed at this life stage. Therefore, NTP1-4 gene was excluded from further analyses.

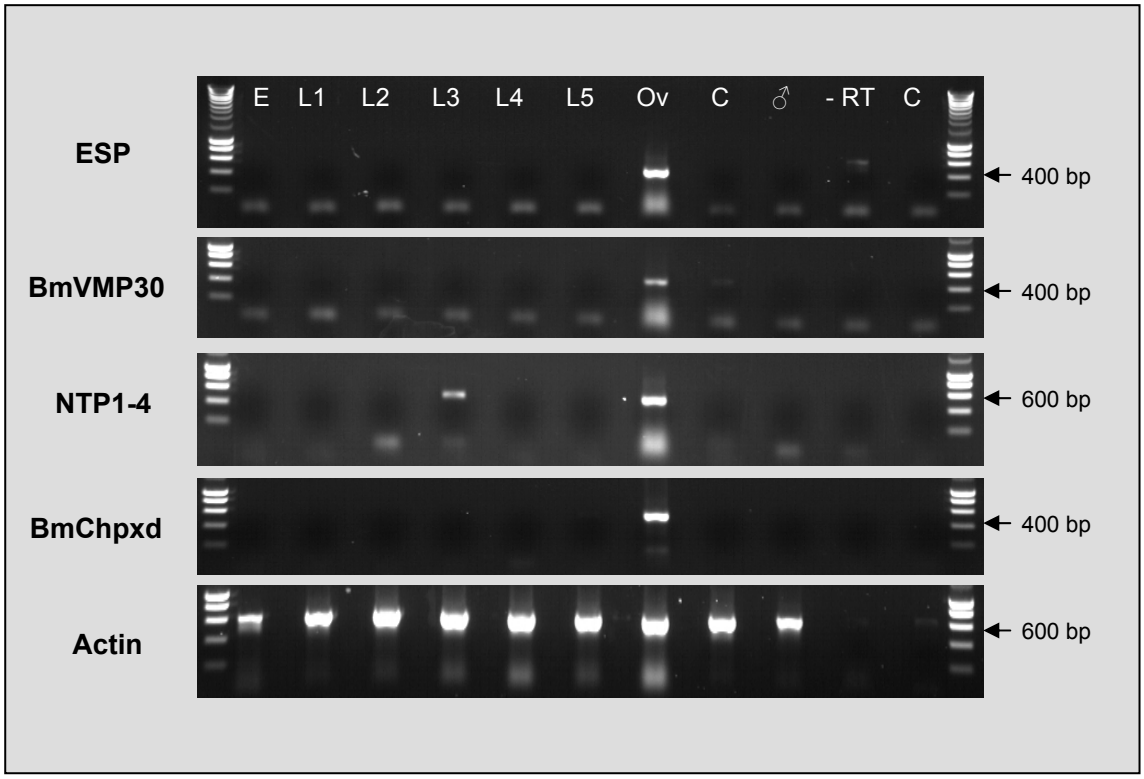

Figure 5.3. Expression profile analysis of egg-specific protein (ESP), vitelline membrane protein 30 (BmVMP30), NTP1-4, and chorion peroxidase (BmChPxd) in *B. mori*. Developmental stages included: E, egg; L1 to L5, five different larval instars; Ov, female ovary; C, female carcass; ♂, male; -RT, no reverse transcriptase negative control; and C, no-cDNA negative control. Primers and expected fragment sizes were ESPF and ESPR (450 bp) for ESP, VMP-RTF and VMP-RTR (595 bp) for BmVMP30, NTPF and NTPR (469 bp) for NTP1-4 and 1130 and 911 (477 bp) for BmChpxd (or 1245 bp for gDNA). A *B. mori* muscle actin gene control was included, amplified with primers 1075 and 1076 and an expected amplicon size of 609 bp. Smart ladder is run at both ends.

Several RT-PCR were performed for Eckinase with different primer pairs, including primers used by Sonobe and colleagues (2006) when characterising the EcKinase gene in *B. mori*. However, RT-PCRs failed to generate the expected product size in any developmental stage. 5' rapid amplification of cDNA ends (5' RACE) was also performed but a PCR product was not obtained, even when the 5' RACE protocol used by Sonobe and colleagues (2006) was followed. Differences between results obtained in this study and those from Sonobe and colleagues might be due to the use of different *B. mori* strains. Although, the hybrid *kinshu* × *showa* strain was not

available, five other strains (Dazao, green, yellow, black and pink strain) were tested in this study. As a result, no further work was carried out with the EcKinase gene.

5.3.3. Use of reporter genes to test putative regulatory sequences

In order to determine whether the putative promoters of the candidate genes could drive the expression of a transgene in the pink bollworm ovary, the putative promoters were amplified from *B. mori* genomic DNA and linked to the coding sequence for a fluorescent protein. Putative promoter sequences were obtained by blasting the gene sequence against the *B. mori* genome sequence. The DNA sequence upstream of the coding region, including the 5' UTR, was considered to be the putative promoter. Between two to 4.5 kb of this sequence was used, hoping that the fragment included all the regulatory sequences necessary for full expression. Primers were designed at the ends of the sequence and the putative promoters were amplified from *B. mori* genomic DNA.

All constructs consist of an *Opie2*-DsRed2 transformation marker and a sequence encoding the green fluorescent protein (ZsGreen) driven by the putative candidate gene promoter, flanked by *piggyBac* cis-acting sequences (Figure 5.4). In addition, both marker genes are flanked by nuclear localisation signals to direct fluorescence to the nuclei of the cells.

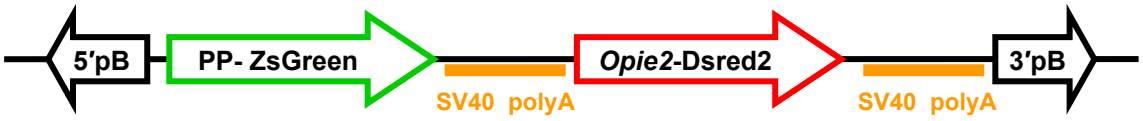

Figure 5.4. General schematic diagram of the marker-only constructs. PP = putative promoter.

The transformation marker is intended to allow for the quick and easy detection of transgenic individuals. The putative promoter-ZsGreen marker allows for observation, by viewing green fluorescence, of the expression pattern of the promoter (in terms of tissue, stage and levels). The only variation between constructs is the putative promoters, unless otherwise stated. Three constructs were built following this design and injected into wild-type pink bollworm embryos (Table 5.1).

Table 5.1. Injection data for OX4270, OX4413 and OX4660. BmChPxd = *B. mori* chorion peroxidase; ESP = egg specific protein; BmVMP30 = *B. mori* vitelline membrane protein 30.

Construct	Putative promoter (size)	No. embryos injected	No. injection survivors	No. transgenic lines	Transformation efficiency (%)
OX4270	BmChPxd (4500 bp)	1951	783	19	2.4
OX4413	ESP (2551 bp)	1442	176	6	3.4
OX4660	BmVMP30 (2099bp)	922	221	4	1.8

Microinjections were carried out as described in Chapter 2, using an injection mix of 500 ng/µl of DNA plasmid and 300 ng/µl of OX265 helper plasmid. Of the injected eggs, 12.2% to 40% survived to pupation. In comparison, Peloquin and colleagues (2002) achieved a 36.5% post-injection survival when they transformed pink bollworm using *piggyBac*. It is also comparable to survival obtained in diamondback moth (Chapters 3 and 4), which ranged from11% to 39%.

The G_0 adults were out-crossed to wild-type counterparts, in pools of 2-5 injection survivors. Resulting progeny were collected as pupae and were examined under a

fluorescent dissecting microscope for expression of red fluorescence generated by the *Opie2*-DsRed2 marker (Figure 5.5).

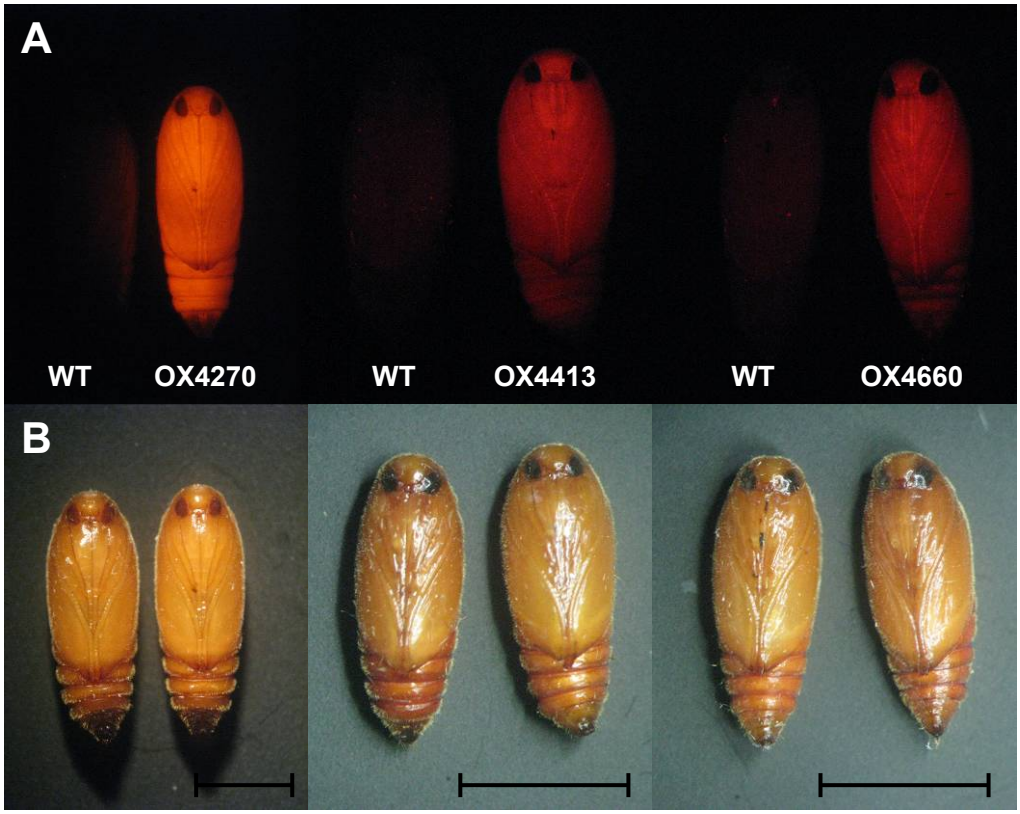

Figure 5.5. DsRed2 expression in wild-type and OX4270T, OX4413G, and OX4660A pupae; under (A) red-excitation wavelength light (B) white light. Scale bar representes 5 mm.

Between four and 19 independent transgenic lines were obtained with the three constructs. Expression patterns of transformation marker (DsRed2) fluorescence expression did not noticeably differ between lines of the same construct. As the injection survivors were pooled, it is not possible to determine the sterility/fertility of the injection survivors, or to calculate the precise transformation rate. The estimated transformation efficiency ranged between 1.8% and 3.4% for the three constructs (see Chapter 3, section 3.2.1).

All transgenic lines of OX4413 and OX4660 were dissected and observed for ZsGreen fluorescence in the ovaries. For OX4270, nine of the 19 lines (A, B, C, D, F,

H, J, L and T) were screened for green fluorescence in the ovaries. The ovaries of at least ten females from each line were dissected and examined under a fluorescent stereomicroscope. For OX4270, three out of the nine lines examined (C, F and T) showed green fluorescence in the ovaries (Figure 5.6). As the *B. mori* chorion peroxidase promoter was used, green fluorescence was expected in the follicle cells of near-mature follicles (follicles close to the anterior end of the ovary), as the gene driven by this promoter - the chorion peroxidase gene - is expressed in the follicle cells during choriogenesis, the last stage of oogenesis (Konstandi et al, 2005). As predicted, ZsGreen expression was confined to almost fully matured follicles. Expression was particularly strong at the posterior end of the follicle, where the follicle cell mono-layer is thicker during this stage of follicle development (Figure 5.6 D).

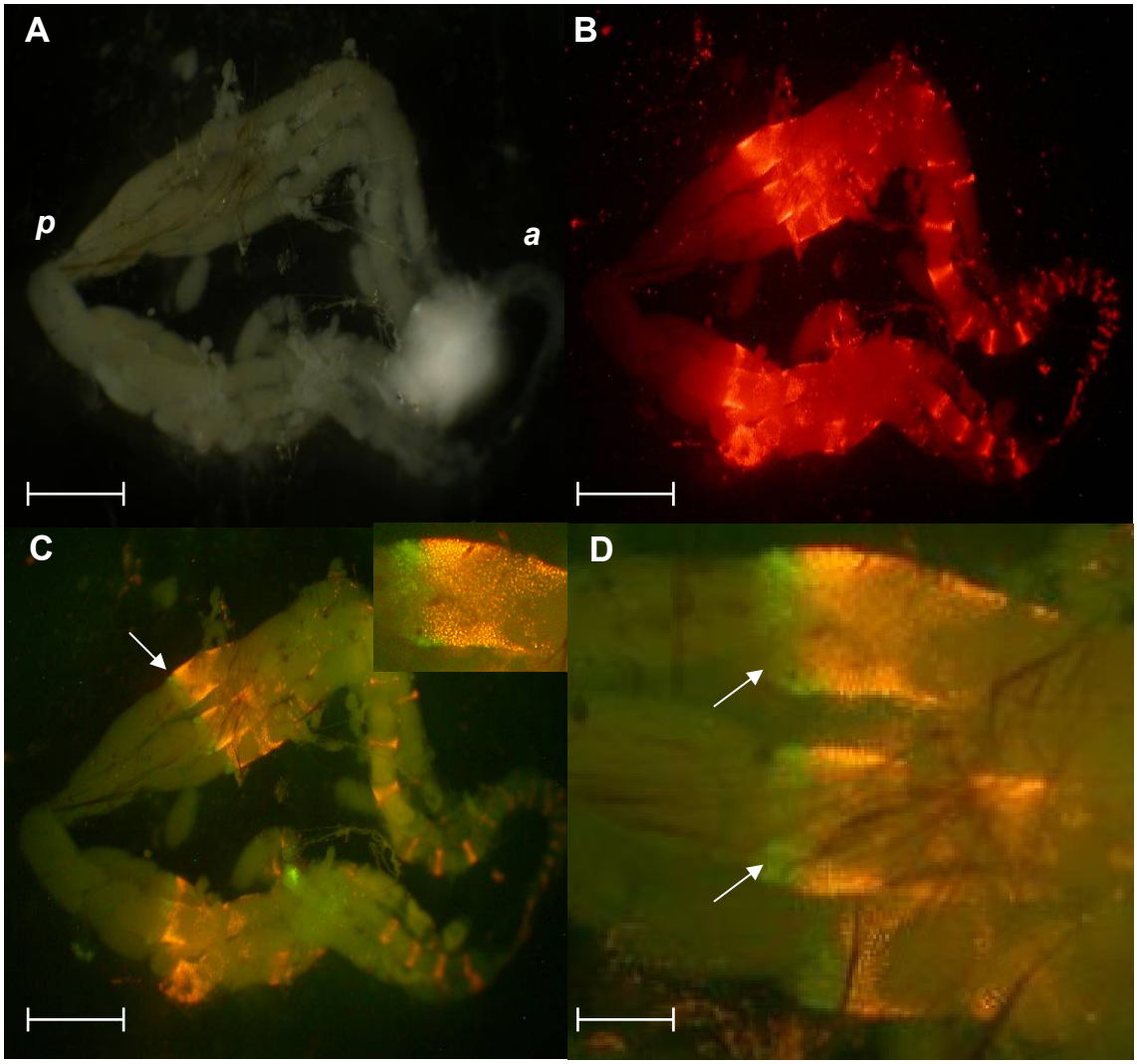

Figure 5.6. Expression of ZsGreen in OX4270T ovaries; under (A) white light; (B) red excitation light showing transformation marker Opie2-DsRed2 expression; and (C) green excitiation light showing green expression in late follicle development (arrow) and magnification of a single follicle (inset). (D) shows the same ovary at higher magnification. Note that some red fluorescence bleeds through to the green filter producing an orange fluorescence. *a* = anterior end of the ovary; *p* = posterior end of the ovary. Scale bar for panles A, B and C represents 50 µm and for panel D represents 10 µm.

Although the three lines expressed ZsGreen in the follicles, there was some variation in the level of expression between lines. Two lines, C and T, showed stronger expression than line F (Figure 5.7). Lines C and T showed a more pronounced green fluorescent band compared to line F. ZsGreen expression was not visible at all in lines A, B, D, H, J and L. Position effect seems to exert a strong influence on the activity of this promoter fragment, at least in the context of this construct.

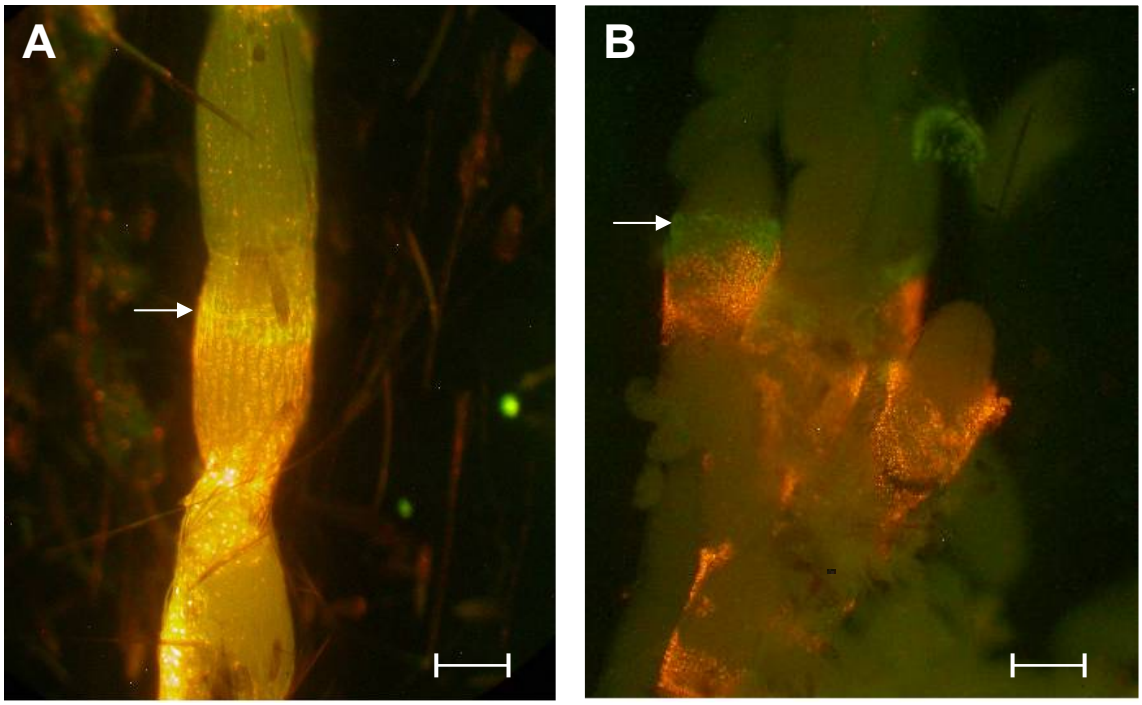

Figure 5.7. Different levels of ZsGreen expression in the follicle cells. (A) line OX4270F with weak green fluorescent expression (arrow); (B) line OX4270C with stronger green fluorescent expression (arrow). DsRed2 bleeds through the green filter, producing orange fluorescence, due to the use of longer exposure to capture the weak green fluorescence. Scale bar represents 5 μm.

For OX4413, four out of the seven lines examined show green fluorescence in the ovaries (A, D, E and G) (Figure 5.8). As expected, based on *B. mori* ESP expression profile, green was detected in the follicle cells of intermediate to near-mature follicles. Compared to the *B. mori* chorion peroxidase, where expression is localised to the nearly matured follicle, expression is observed in almost every follicle, with the exception of most immature and mature follicles at the posterior and anterior end of the ovary, respectively (Figure 5.8.C). ZsGreen expression becomes stronger at the posterior end, as follicles mature and has a thicker layer of follicle cells.

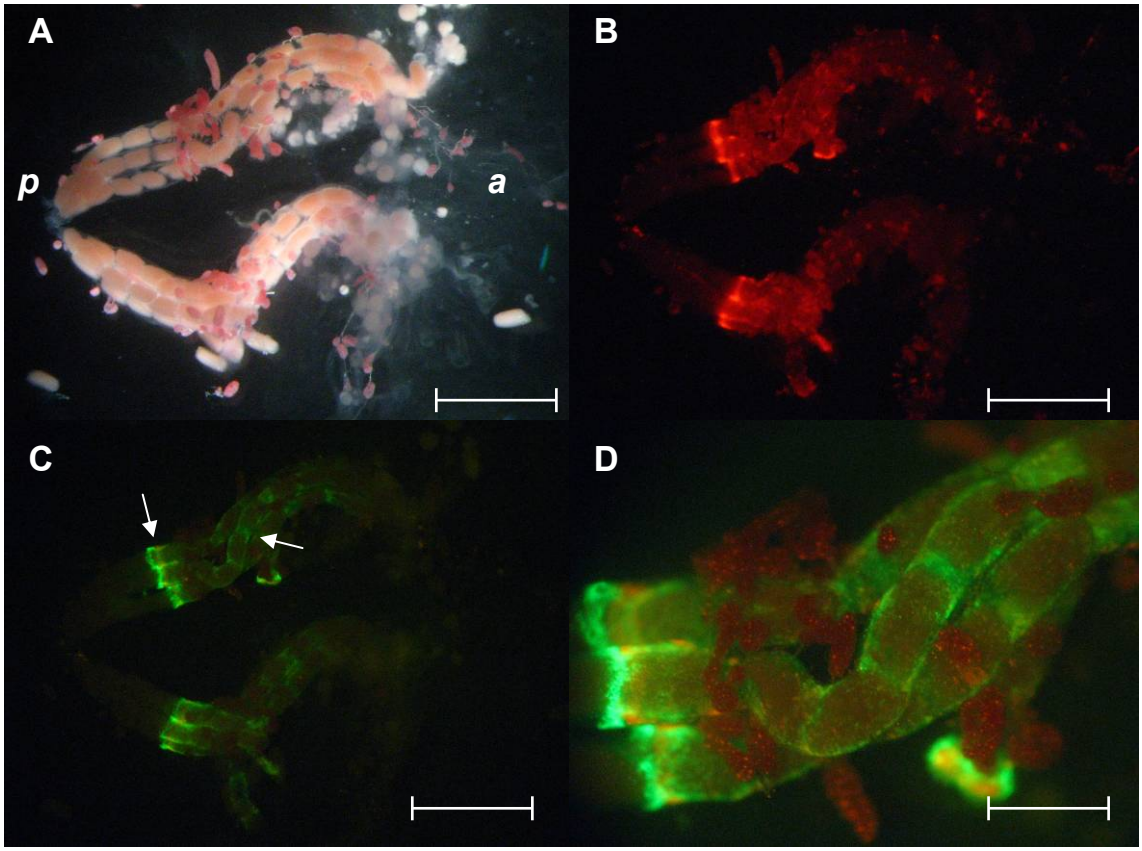

Figure 5.8. Expression of ZsGreen in the OX4413D female ovary; under (A) white light; (B) red excitation light showing red expression of the *Opie2*-DsRed2 transformation marker; and (C) green excitation light showing green expression in different developmental stage follicle (arrows). (D) shows the same ovary at higher magnification. *a* = anterior end of the ovary; *p* = posterior end of the ovary. Scale bar for panels A to C represents 50 μm and for panel D represents 10 μm.

For OX4660, all lines expressed green fluorescence in the ovaries. Green expression was detected in follicle cells of nearly matured follicles (Figure 5.9), in a very similar way to the *B. mori* chorion peroxidase. This was to be expected as both the *B. mori* chorion peroxidase and BmVMP30 genes are expressed almost simultaneously at the beginning of choriogenesis.

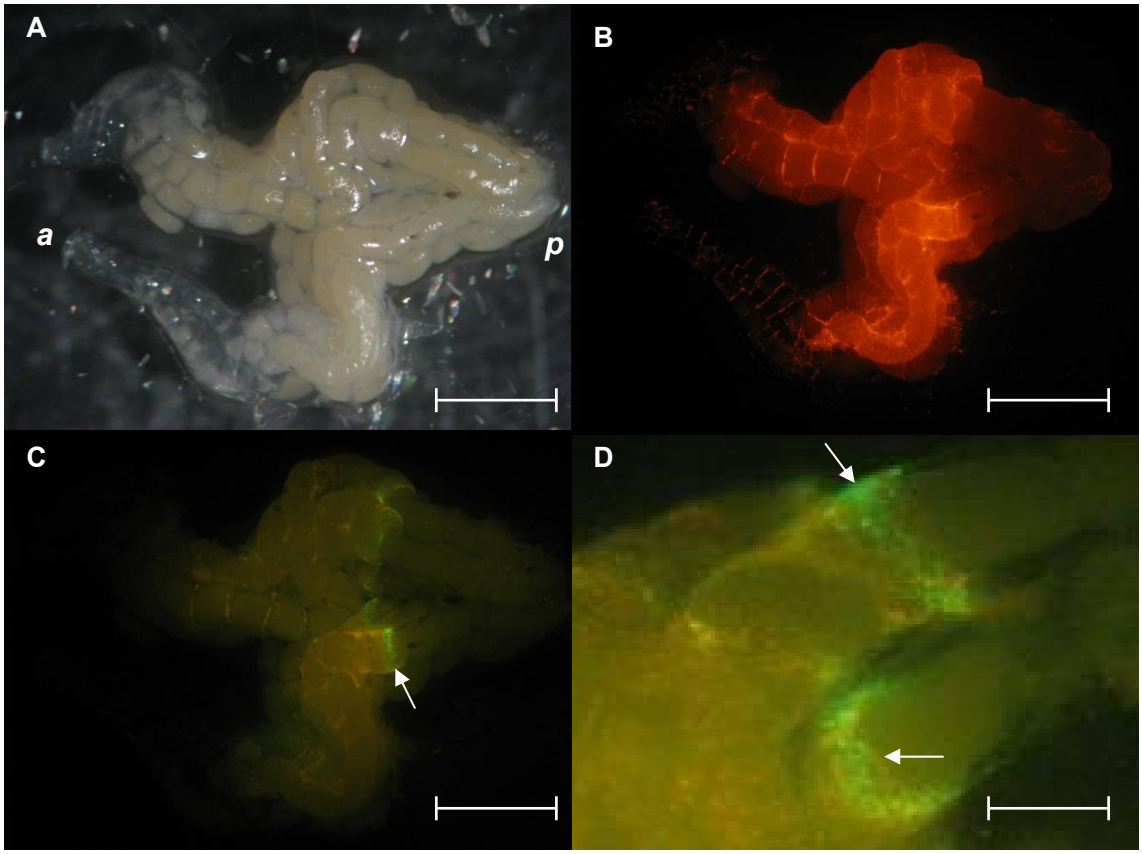

Figure 5.9. Expression of ZsGreen in the OX4460A ovaries; under (A) White light; (B) red excitation light showing red expression of the *Opie2*-DsRed2 transformation marker; and (C) green excitation light showing green expression in different developmental stage follicle (arrow). (D) shows the same ovary at higher magnification. *a* = anterior end of the ovary; *p* = posterior end of the ovary. Scale bar for panels A to C represents 50 µm and for panel D represents 5 µm.

For all lines examined, the remaining female carcass was examined for ZsGreen expression in order to determine if the promoter drove the expression of the green marker in other body tissues besides the follicular epithelium. ZsGreen expression was not observed in any other body part. Male adults were also examined for ZsGreen fluorescence to ensure expression was restricted to the females. In this study, true marker fluorescence was determined by the punctate appearance caused by the use of the nuclear-localisation signal that directs the fluorescence protein to the cell nucleus where it accumulates. However, to check the possibility of green auto-fluorescence -

background fluorescence emitted by the insect tissues – in wild-type ovaries, they were observed in parallel, and no such fluorescence was observed.

5.3.4. Development of tTAV driver strains

B. mori putative gene promoters were shown to induce temporal and tissue-specific expression of a reporter gene in pink bollworm ovaries. In order to determine if such promoters could be used to drive tissue-specific lethality in the ovaries, more exactly follicle cells, new constructs were designed to express the tetracycline transactivator tTAV under the regulation of the candidate putative promoter. Transformed lines were then crossed with other lines carrying the *tetO* sequence combined with a fluorescence protein or lethal effector gene.

All constructs consisted of a *piggyBac* transposon vector comprising the tetracycline transactivator tTAV being driven by one of the putative promoters, a Hr5*ie1*-ZsGreen transformation marker and two 3'-UTR from SV40 (Figure 5.10). The ZsGreen gene is flanked by two nuclear localisation signals. The only difference between the three constructs designed is the putative promoter.

Figure 5.10. General schematic diagram of the tTAV constructs. PP = putative promoter.

All constructs were co-injected with OX265 helper plasmid, a source of transposase, in wild-type pink bollworm embryos. A total of 1114 to 1700 embryos were injected,

depending on the construct (Table 5.2). From this 16.3% to 35.4% survived to pupal stage and were crossed, in pools of 2-5 survivors, with wild-type mates. Progeny was screened for *Opie2*-ZsGreen marker expression. Four, three and 11 independent lines were obtained for OX4630, OX4498 and OX4521, respectively (Figure 5.11). Lines were named OX4630A-D, OX4498A-C and OX4520A-K. Differences in marker expression were not detected between lines of the same construct.

Table 5.2. Injection data for OX4360, OX4498 and OX4521 tTAV driver strains. BmChPxd = *B. mori* chorion peroxidase; ESP = egg specific protein; BmVMP30 = *B. mori* vitelline membrane protein 30.

Construct	Putative promoter	No. of embryos injected	No. of injection survivors	No. of transgenic lines	Transformation efficiency (%)
OX4360	BmChPxd	1114	182	4	2.2%
OX4498	BmVMP30	1682	684	3	0.4%
OX4521	ESP	1700	601	11	1.8%

The founder transgenic individuals of one OX4498 line (OX4498C) and three OX4521 lines (OX4521E, H and I) did not produce any progeny, and the lines were lost. This may have simply been due to the transgene insertion disrupting the function of an endogenous gene.

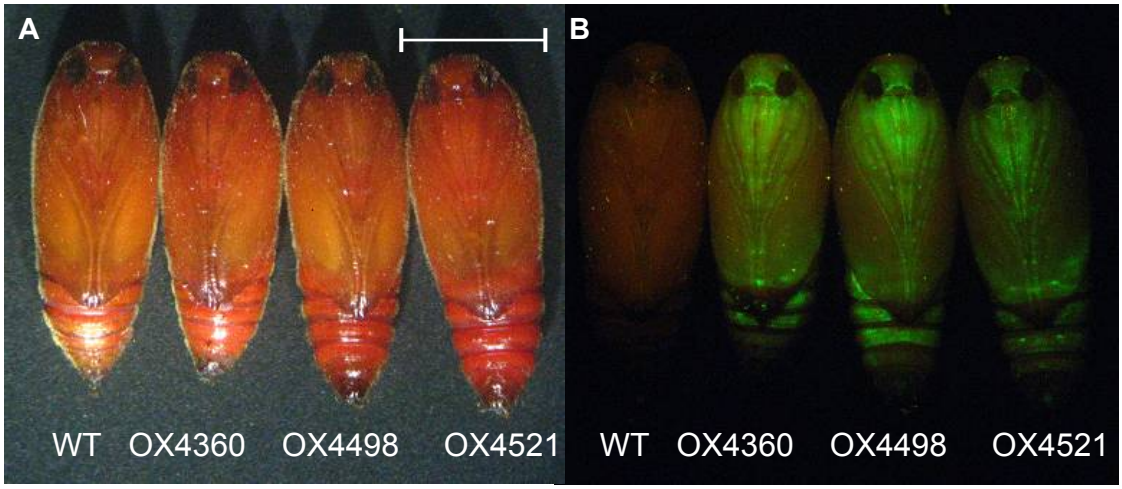

Figure 5.11. DsRed2 expression in wild-type and OX4360AT, OX4498A, and OX4521B pupae; (A) white light (B) green fluorescence. Scale bar represents 3 mm.

5.3.5. Crossing tTAV driver strains with a *tetO*-AmCyan reporter strain

To determine if putative promoter driven tTAV could induce expression of a *tetO*-effector gene, the OX4360, OX4498 and OX4521 tTAV driver lines were crossed to a *tetO*-effector gene strain, OX4026.

The OX4026 consists of 14 copies of *tetO* sequence, *hsp70* mini promoter driving the expression of the AmCyan fluorescent gene, and an *Opie2*-DsRed2 transformation marker (Figure 5.12). The DsRed2 gene is flanked by two nuclear localisation signals. Two OX4026 lines, A and D, had been previously generated and tested by colleagues.

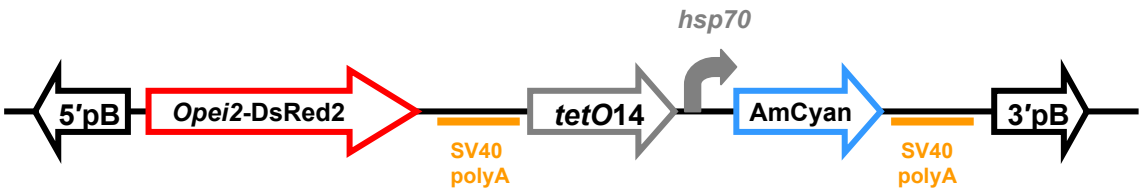

Figure 5.12. Schematic diagram of OX4026 construct.

In progeny of driver/effector strain crosses that carry both transgenes, the tTAV driven by the putative promoter should bind to the *tetO* sequence. As designed, this should enhance expression of the AmCyan fluorescent protein, in the ovaries where the putative promoter is active, when the insects are reared in the absence of tetracycline. On the contrary, AmCyan expression should not be detected when the insects are reared on tetracycline (Figure 5.13).

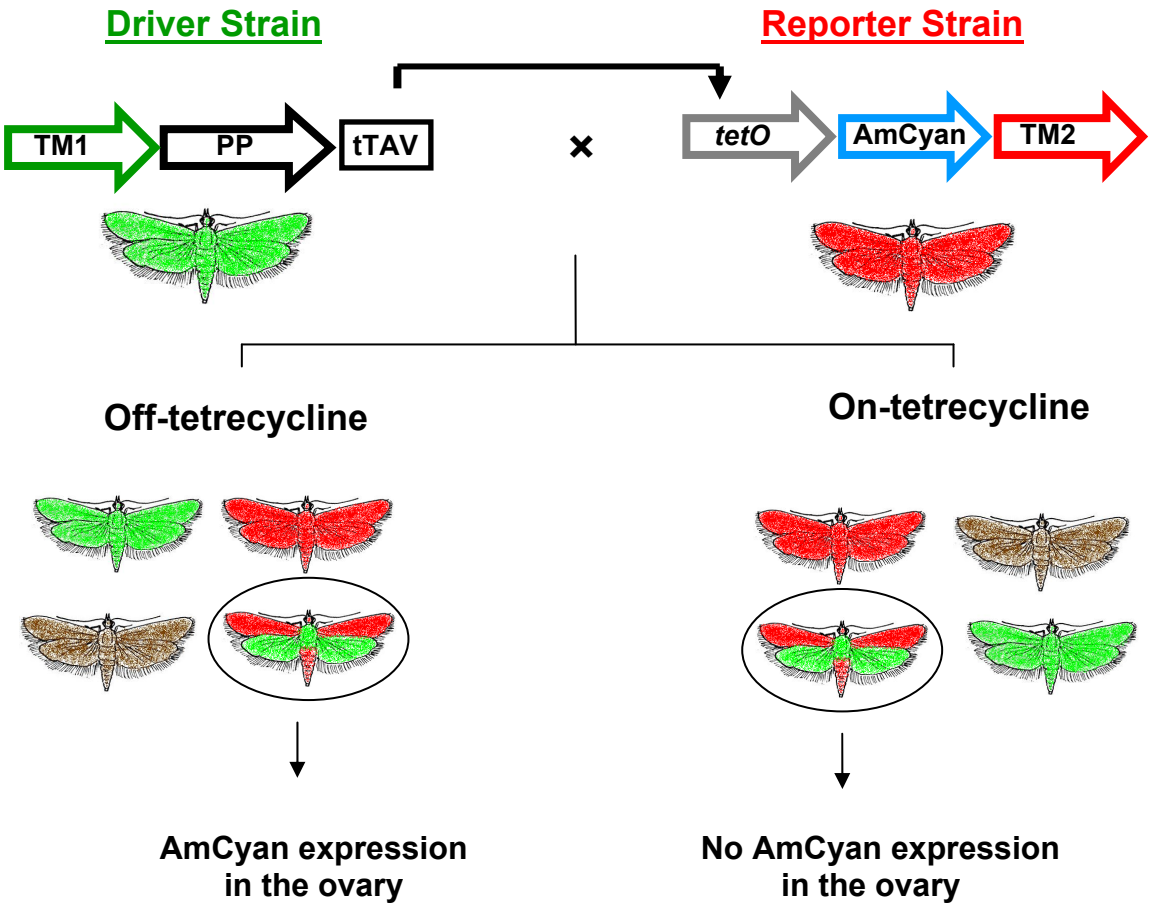

Figure 5.13. Scheme showing the genetic cross between individuals, carrying a dominant allele, of a driver and a reporter strain. TM = transformation marker (TM1-green and TM2-red); PP = putative promoter. Resulting progeny consist of 25% of wild-type individuals (brown moth), 25% of individuals carrying only the driver strain transgene (green moth), 25% of individuals carrying only the reporter strain transgene (red moth) and 25% of individuals carrying both driver and reporter strain transgenes (red and green moth). Female moths carrying both transgenes and reared off tetracycline should express Amcyan in their ovaries. In contrast, females carrying in both transgenes and reared on tetracycline should not express AmCyan in their ovaries as tTAV binds to the tetracycline instead to be *tetO* sequence and the expression of the AmCyan gene is prevented.

Groups of 10 driver strain individuals from OX4360, OX4498 and OX4521 lines were crossed to OX4026A and D mates, in a ratio of 1♂:3♀. Progeny was divided into two groups: one reared on tetracycline and the other off-tetracycline, throughout their development. Ovaries from at least 5-day-old females, carrying both driver (green-marked) and reporter (red-marked) transgenes and reared on and off tetracycline, were dissected and examined for AmCyan expression under a fluorescence microscope. AmCyan expression was not detected in the ovaries of female progeny derived from the cross of any of the OX4360 and OX4498 to OX4026A and OX4026D lines. In this context, ovaries expressed AmCyan in all OX4521 lines (Figure 5.14), except OX4521D and OX4521F. In addition, AmCyan was not detected in progeny counterparts reared on tetracycline, indicating that the system is tetracycline-repressible (Figure 5.15). These results provide an indication that the OX4521 strains that showed function here are most likely to affect female fertility or egg viability, in a conditional manner, when AmCyan is replaced by a lethal effector gene.

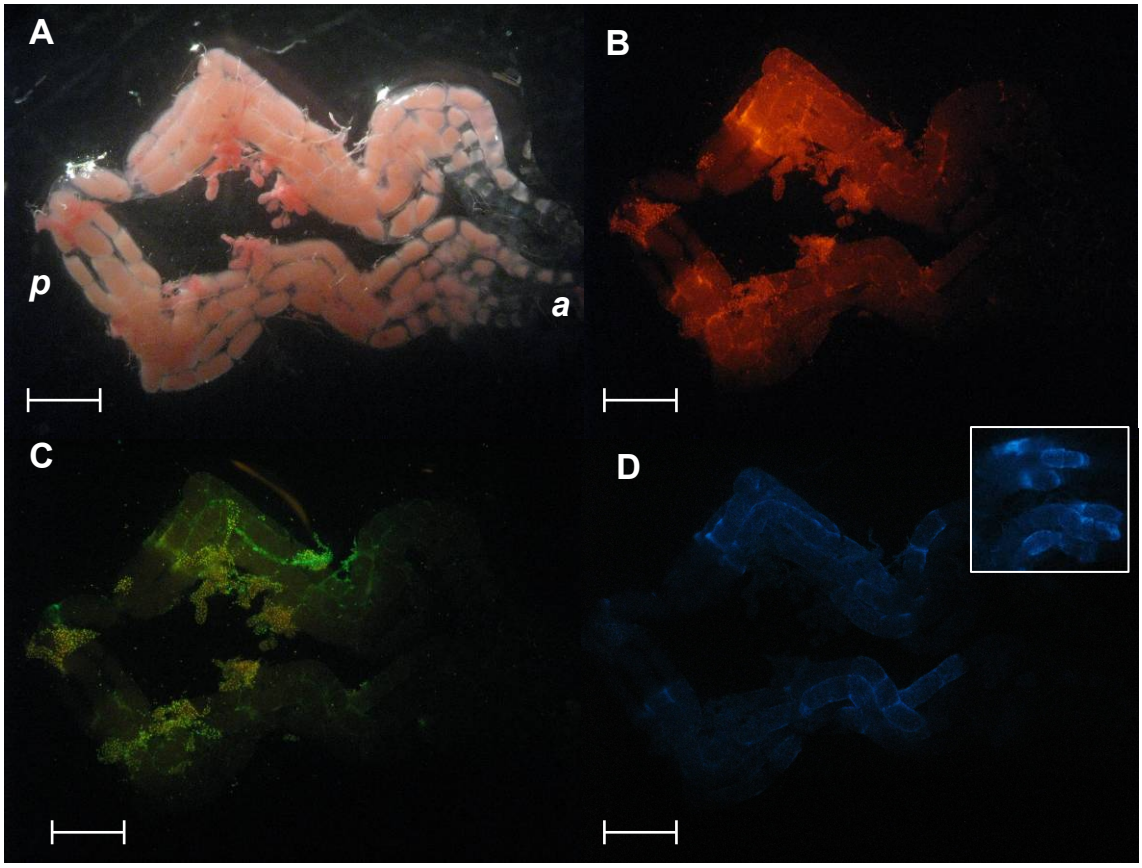

Figure 5.14. Expression of AmCyan in the OX4521B ovary of a female, reared off tetracycline; under (A) white light; (B) red excitation light showing the OX4026D reporter strain red transformation marker; (C) green excitation light showing the OX4521B driver strain green transformation marker; (D) blue excitation light showing cyan expression in most follicles. Inset, magnification of follicles. *a* = anterior end of the ovary; *p* = posterior end of the ovary. Scale bar represents 50 μm.

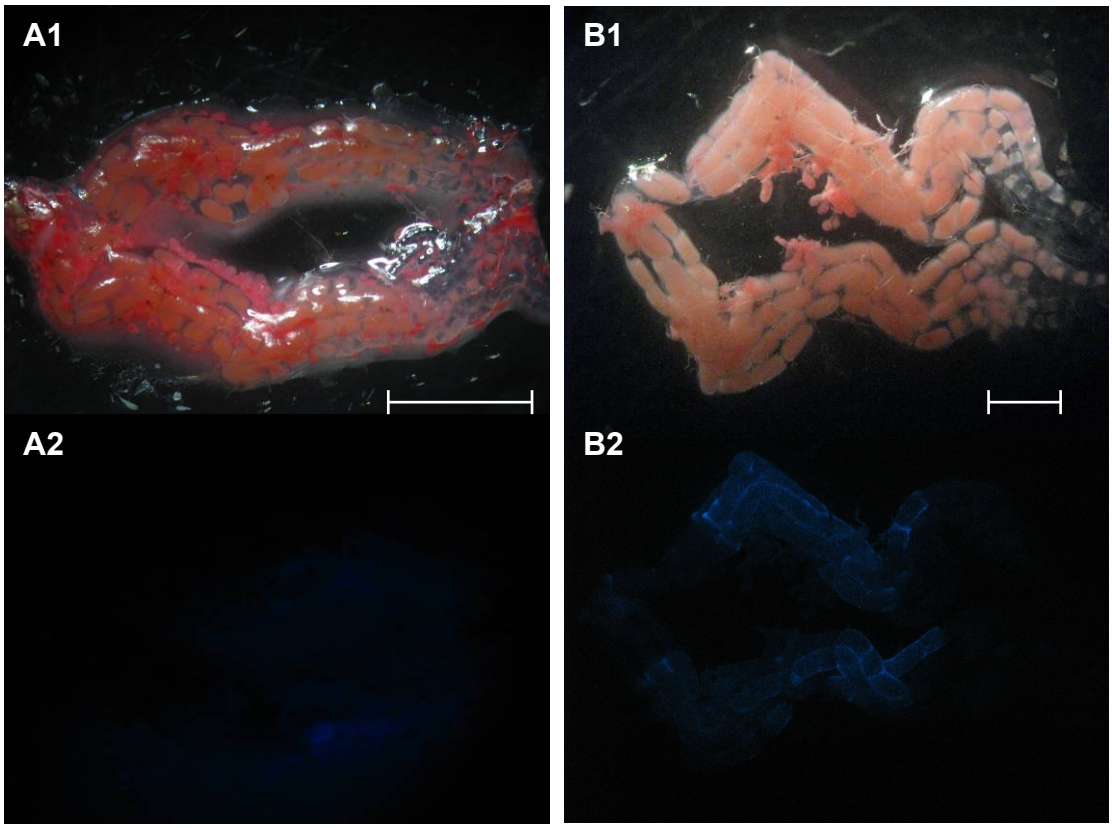

Figure 5.15. **Expression of AmCyan in the OX4521B ovary of a female**; reared (A) on and (B) off -tetracycline, under (1) white light and (2) blue excitation. Scale bar represents 50 μm.

5.3.6. Crossing tTAV driver strains with a *tetO-reaper*^{KR} strain

In parallel with the crosses to the *tetO*-AmCyan reporter strain (OX4026), crosses between the different OX4360, OX4498 and OX4521 driver lines and a lethal effector strain, carrying *tetO* linked to a lethal effector gene instead of a fluorescent protein gene, were also set up. These crosses were performed in order to determine if driver lines could drive ovary-specific expression of a lethal gene. Several lethal genes have previously been tested in pink bollworm by colleagues. These include *reaper*^{KR}, NIPP1Dm and calmodulin^{B34}. However, *reaper*^{KR} was the only one to induce lethality when strains carrying the ubiquitous *B. mori* heat shock protein 83 (*Bmhsp83*) driving

tTAV were crossed to a *tetO*-effector strain (data not shown). Therefore, *reaper*^{KR} was selected as the effector gene to be tested in this study.

reaper is a pro-apoptotic gene, of which *reaper*^{KR} is a mutated variant (Olson et al. 2003). The mutation of lysines (K) to arginines (R) prevents ubiquitination of the protein, conferring resistance to degradation, and therefore greater persistence in cells. In *D. melanogaster*, it has been shown that *reaper*^{KR} acts by binding to the *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1), thereby inhibiting DIAP1 function, which is followed by the activation of the primary cell death executioners, the caspases (Zhai et al. 2009). Reaper is expressed in the cytoplasm of cells destined to die (Wing et al. 1998, Bryant et al. 2009). The OX3069C strain, generated by colleagues, carries seven *tetO* copies and the *heat-shock protein 70* (*hsp70*) minimal promoter driving the expression of the *reaper*^{KR} gene. It also contains an *Opie2*-DsRed2 transformation marker (Figure 5.16).

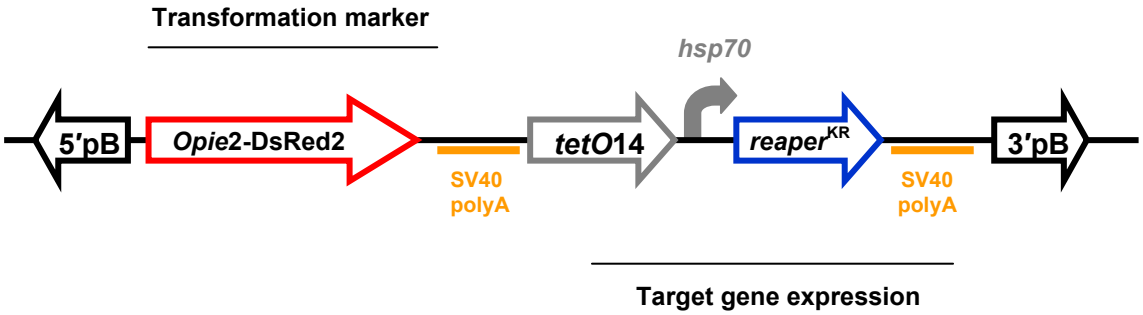

Figure 5.16. Schematic diagram of OX3069. pB = *piggyBac* terminal repeats

As with OX4026, it was expected that, after crossing the driver lines with OX3069C, in progeny carrying both transgenes the putative promoters would drive the expression of the tTAV protein, which would bind to the *tetO* enhancer in OX3069. To provide sterility, consequent expression of the *reaper*^{KR} would need to result in

death or functional incapacitation of the follicular cells in the ovaries and thereby render females sterile. This system was designed to be repressible in the presence of tetracycline provided in larval diet.

To test the feasibility of this system, for all OX4360, OX4498 and OX4521 driver lines hemizygous individuals were crossed with OX3069C hemizygous mates. Progeny were divided into two groups, one reared off tetracycline and one on tetracycline diet. As pupae, they were divided into the four possible phenotypes: pupae carrying both driver and effector strain transgenes (red and green transformation markers); pupae carrying only the driver transgene (green only); pupae carrying only the effector transgene (red only), and wild-type (no marker).

Table 5.3 shows the observed number for each phenotype for OX4360 driver lines crossed to OX3069C. With no survival advantage for any one genotype, the four different phenotypes should be in a ratio of 1:1:1:1. For the OX4360 lines, Chi-squared analysis (d.f=3 and $p>0.05$) of the observed numbers shows that the ratio between the different phenotypes is not significantly different from 1:1:1:1, when insects were reared on non-tetracycline diet: OX4360A (χ^2=1.696, p=0.6377), OX4360B (χ^2=0.286, p=0.9607), OX4360C (χ^2=0.424, p=0.9353), and OX4360D (χ^2=0.600, p=0.8964). Phenotype ratios of insects reared on tetracycline did not differ significantly from the expected 1:1:1:1 ratio: OX4360A (χ^2=0.712, p=0.8703), OX4360B (χ^2=0.408, p=0.9386), OX4360C (χ^2=0.370, p=0.9463), and OX4360G (χ^2=950, p=0.8133).

Table 5.3. Summary of each phenotype in progeny from OX4360 lines crossed to OX3069C, reared off (NT) and on (T) tetracycline food. R+G = individuals carrying both OX4360 and OX3069 transgenes; G = individuals carrying only the OX4360 transgene; R = individuals carrying only the OX3069C transgene; WT= wild-type.

	Fluorescence phenotype							
	Line	NT				T			
		R+G	G	R	WT	R+G	G	R	WT
OX4360	A	56	60	61	70	68	72	74	78
	B	88	95	90	91	78	77	82	84
	C	73	77	78	81	69	73	76	71
	D	70	65	71	74	67	70	67	77

For the OX4498 lines (Table 5.4), Chi-squared analysis (d.f.=3 and $p>0.05$) of the observed numbers shows that the ratio between the different phenotypes is not significantly different from 1:1:1:1, when insects were reared on non-tetracycline diet: OX4498A (χ^2=0.689, p=0.7858) and OX498B (χ^2=0385, p=0.9434). Phenotype ratios of insects reared on tetracycline did not differ significantly from the expected 1:1:1:1 ratio: OX4498C (χ^2=1.040, p=0.7916) and OX4498D (χ^2=0.196, p=0.93782).

Table 5.4. Summary of each phenotype in progeny from OX4498 lines crossed to OX3069C, reared off (NT) and on (T) tetracycline food. R+G = individuals carrying both OX4498 and OX3069 transgenes; G = individuals carrying only the OX4498 transgene; R = individuals carrying only the OX3069C transgene; WT= wild-type.

	Fluorescence phenotype							
	Line	NT				T			
		R+G	G	R	WT	R+G	G	R	WT
OX4498	A	55	58	63	62	57	64	68	61
	B	49	51	53	55	56	52	55	56

For the OX4521 lines (Table 5.5), Chi-squared analysis (df=3 and $p>0.05$)) of the observed numbers shows that the ratio between the different phenotypes is not

significantly different from 1:1:1:1, when insects were reared off tetracycline diet: OX4521A (χ^2=2.247, p=0.5227), OX4521B (χ^2=0.699, p=0.8733), OX4521C (χ^2=0.389, p=0.9426), OX4521D (χ^2=1.605, p=0.6583), OX4521F (χ^2=0.292, p=0.9616), OX4521G (χ^2=0.240, p=0.9709), OX4521J (χ^2=1.603, p=0.6587) and OX4521K (χ^2=0.387, p=0.9429).

Phenotype ratios of insects reared on tetracycline did not differ significantly from the expected 1:1:1:1 ratio: OX4521A (χ^2=0.523, p=0.9138), OX4521B (χ^2=0.515, p=0.9156), OX4521C (χ^2=0.359, p=0.9486), OX4521D (χ^2=0.462, p=0.9273), OX4521F (χ^2=0.444, p=0.9310), OX4521G (χ^2=0.449, p=0.9299), OX4521J (χ^2=2.230, p=0.5260) and OX4521K (χ^2=0.818, p=0.8451). Results indicate that the presence of both transgenes in the genome did not cause significant lethality in these experiments.

Table 5.5. Summary of each phenotype in progeny from OX4521 lines crossed to OX3069C, reared off (NT) and on (T) tetracycline food. R+G = individuals carrying both OX4521 and OX3069 transgenes; G = individuals carrying only the OX4521 transgene; R = individuals carrying only the OX3069C transgene; WT= wild-type.

		Fluorescence phenotype							
	Line	NT				T			
		R+G	G	R	WT	R+G	G	R	WT
OX4521	A	98	85	100	105	101	93	95	101
	B	75	83	84	84	106	102	96	100
	C	110	102	107	103	97	93	101	99
	D	66	71	73	81	77	79	79	81
	F	91	88	93	95	87	92	92	96
	G	85	88	86	91	83	91	87	84
	J	66	73	65	78	71	75	83	88
	K	101	105	105	110	91	94	103	97

In order to determine whether the presence of both transgenes in the genome (double-hemizygosis) affects fecundity, when insects were reared off tetracycline, the number

of eggs produced by double-hemizygous females (those carrying one copy of each construct) was counted for each driver line when crossed to OX3069C, based on 10 females for each phenotypic group. Double-hemizygous females reared on tetracycline, and hemizygous females for the two remaining fluorescent phenotypes, were included as controls. A wild-type control, consisting of females from our wild-type stock, was also included as the reference point for egg production and hatch rates.

A one-way ANOVA test showed that fecundity of non-tetracycline OX4360A/OX3069C females was not significantly different to that of wild-type females and remaining control groups ($F_{4,45}$=1.131, p=0.354). The same was observed for OX4360B ($F_{4,45}$=1.328, p=0.274) and OX4360D ($F_{4,45}$=0.832, p=0.512). For OX4360C, the mean number of eggs laid per non-tetracycline OX4360C/OX3069C females carrying both transgenes was significantly lower than that of tetracycline-reared females carrying both transgenes ($F_{4,45}$=5.128, p=0.02, followed by a Dunnett's T3 *post hoc* test) (Figure 5.17 and Figure 5.18) (raw data for these tests can be found in appendix 10).

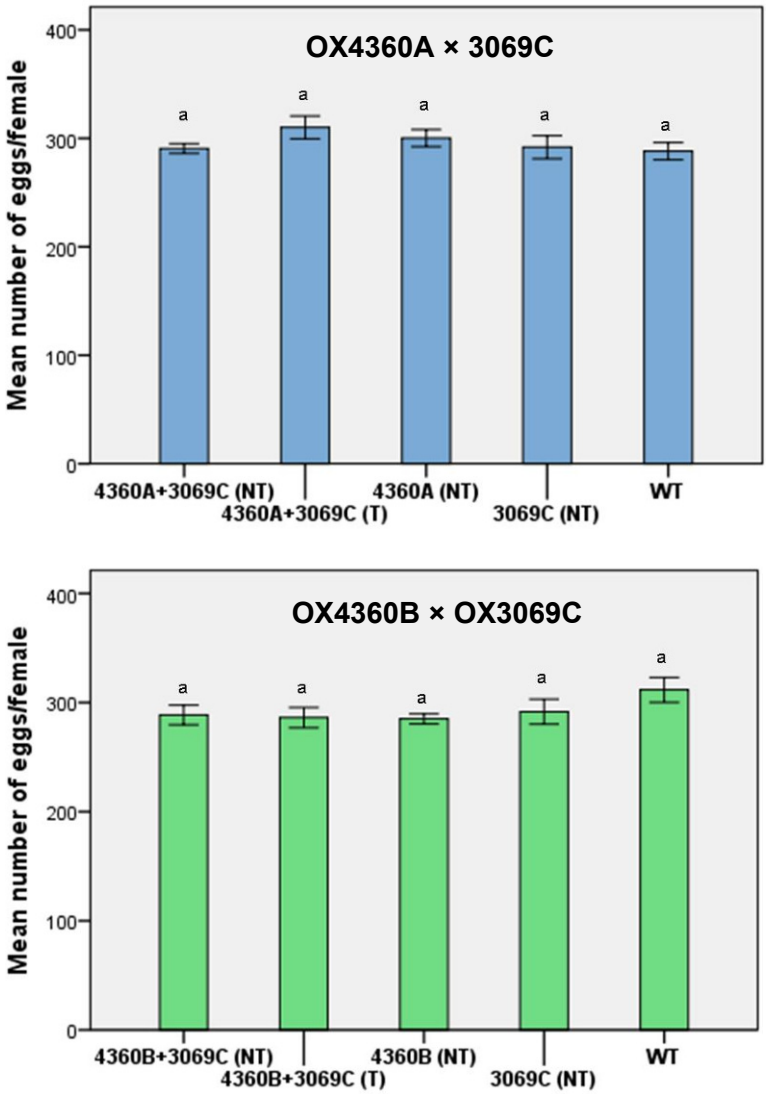

Figure 5.17. **Number of eggs produced per female carrying one, both or neither OX4360A and B and OX3069C transgenes, reared off (NT) or on tetracycline (T).** For all groups *n*=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

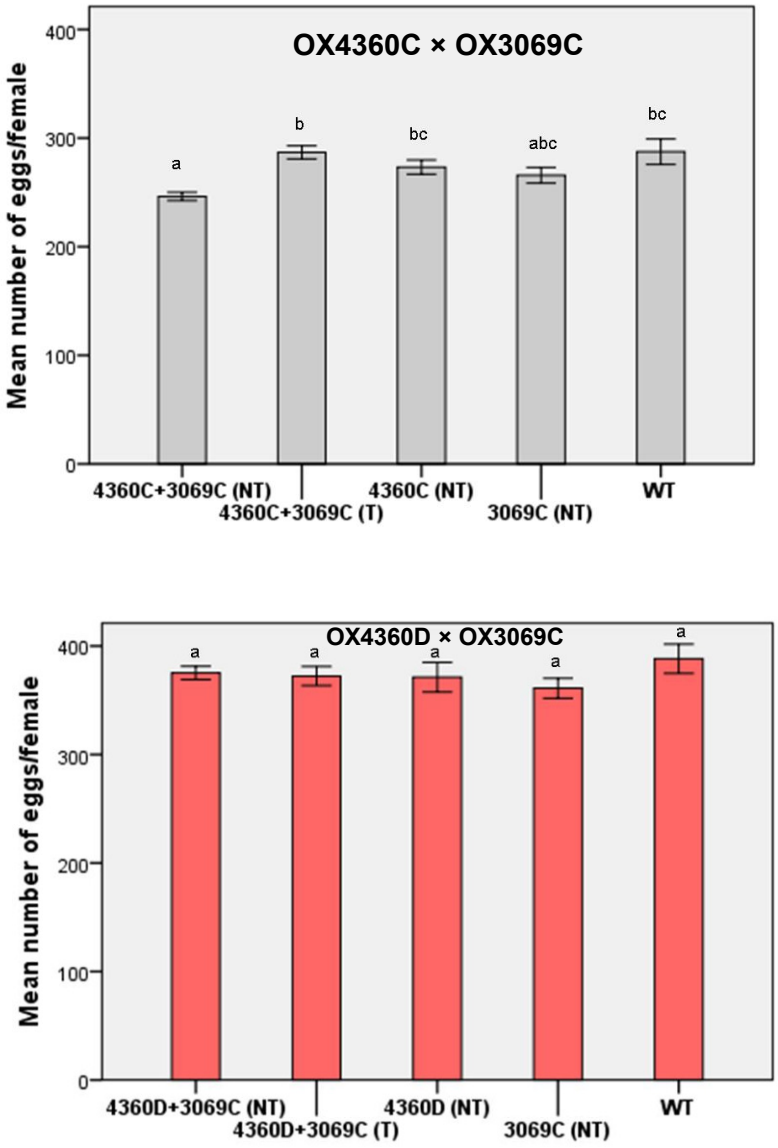

Figure 5.18. Number of eggs produced per female carrying one, both or neither OX4360C and D, and OX3069C transgenes, reared off (NT) or on tetracycline (T). For all groups *n*=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

For the OX4498A, the one-way ANOVA test shows that there were no statistically significant differences in the number of eggs produced per female among phenotypes ($F_{4,45}$=2.551, p=0.052). However, OX4498B/OX3069C females reared on and off tetracycline produced significantly fewer eggs that the remaining phenotypes. This indicates that, in this context, the system may not be tetracycline repressible and/or

the presence of the two transgenes may hinder egg-laying. Without tetracycline-repressibility it is difficult to determine the exact cause (Figure 5.19).

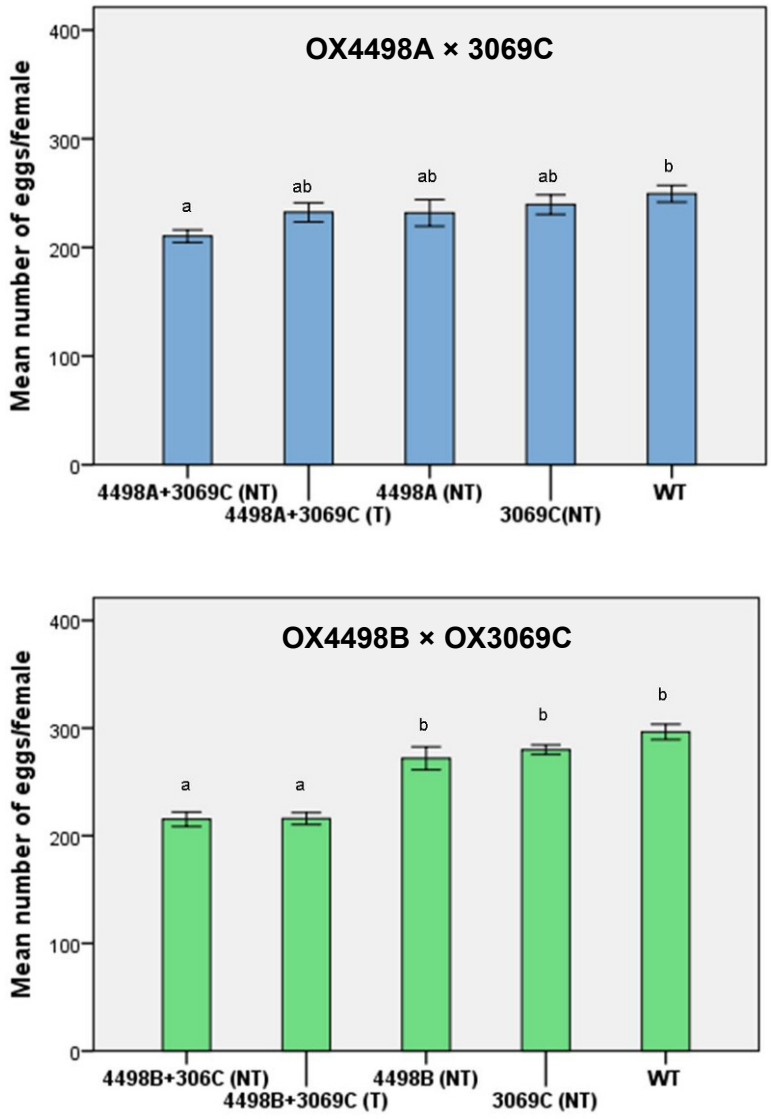

Figure 5.19. Number of eggs produced per female carrying one, both or neither OX4498A and B, and OX3069C transgenes, reared off (NT) or on tetracycline (T). For all groups *n*=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other

The mean number of eggs produced per female of the different OX4521 lines crossed to OX3069C lines is shown in Figure 5.20, Figure 5.21 and Figure 5.22. A one-way

ANOVA test showed that egg production of non-tetracycline OX4521A/OX3069C females was not significantly different to that of wild-type females and remaining control groups: OX4521A ($F_{4,45}$=2.074, p=0.100), OX4521C ($F_{4,45}$=1.001, p=0.417), OX4521D ($F_{4,45}$=0.212, p=0.930), OX4521F ($F_{4,45}$=0.556, p=0.695), and 4521G ($F_{4,45}$=1.139, p=0.350). For the remaining OX4521B, J and K lines, a one-way ANOVA followed by a *post hoc* Dunnett's T3 test showed that in these lines there were some significant differences between some phenotypic groups. In OX4521B ($F_{4,45}$=36.739, p<0.05) and OX4521J ($F_{4,45}$=8.359, p<0.05), the number of eggs produced per female reared off-tetracycline and carrying both transgenes was significantly lower than that of the remaining groups. In OX4521K ($F_{4,45}$=31.881, p<0.05) the average number of eggs produced per off-tetracycline female carrying both transgenes was significantly higher than that of the wild-type group. As we do not anticipate the transgene insertions to increase fecundity, this result is likely due to chance; inspection of the data suggests that this was due to aberrantly low fecundity of the wild type females used.

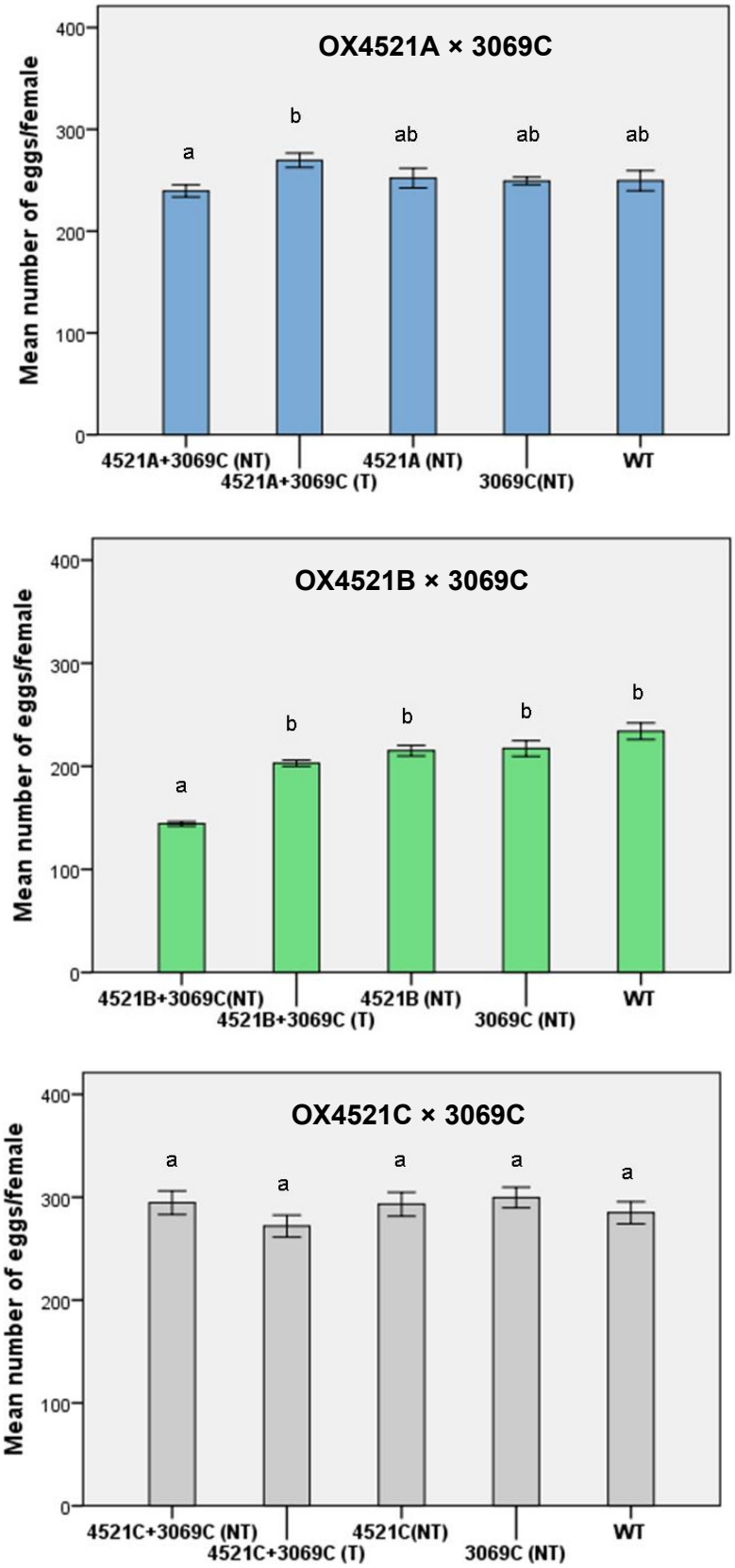

Figure 5.20. Number of eggs produced per female carrying one, both or neither OX4521A, B and C, and OX3069C transgenes, reared off (NT) or on tetracycline (T). For all groups *n*=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

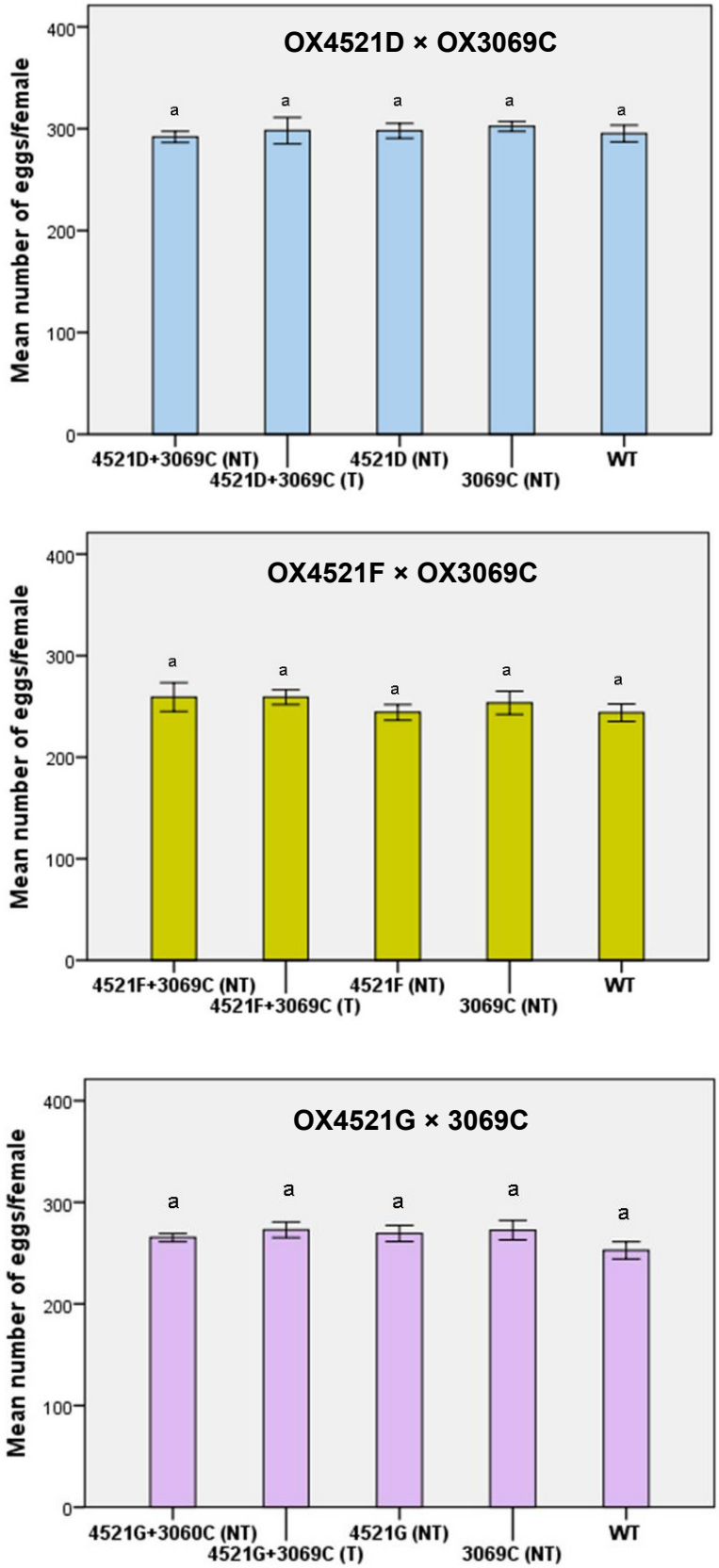

Figure 5.21. Number of eggs produced per female carrying one, both or neither OX4521D, F and G, and OX3069C transgenes, reared off (NT) or on tetracycline (T). For all groups *n*=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

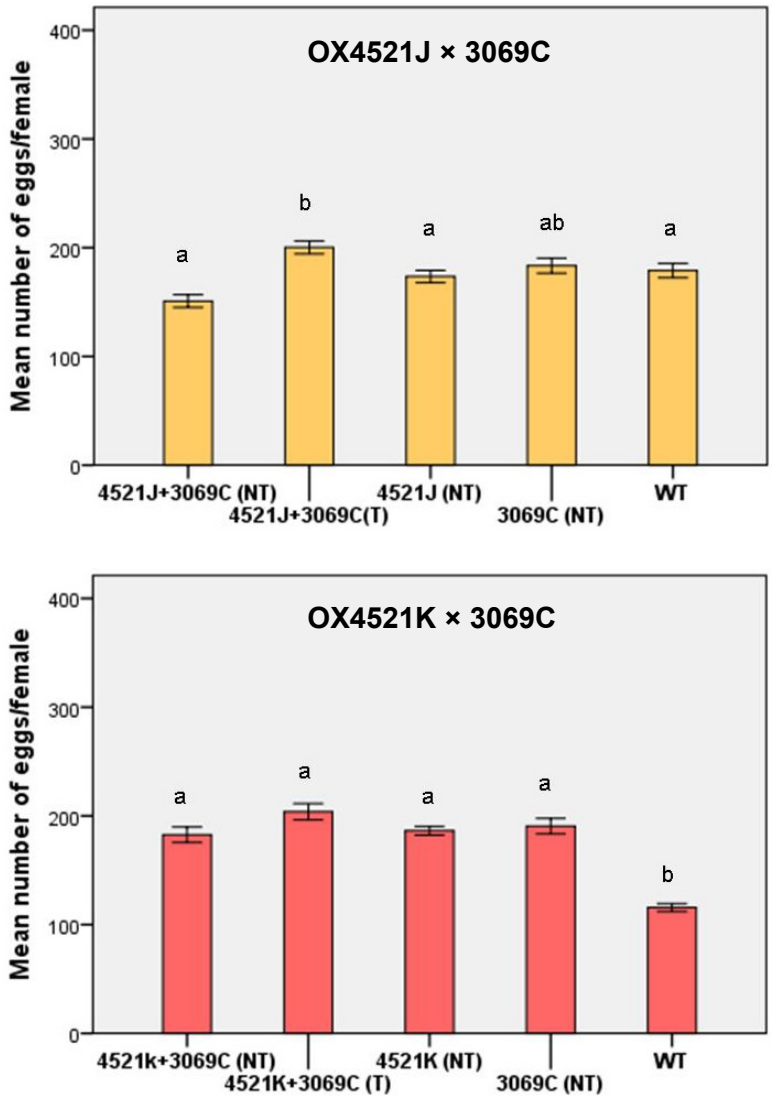

Figure 5.22. Number of eggs produced per female containing one, both or neither OX4521J and K, and OX3069 transgenes, reared off (NT) or on tetracycline (T). For all groups *n*=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

Egg hatch rate was also compared between groups. Eggs produced by each group for each driver and effector strain cross were kept until the eggs hatched. The number of hatched eggs was counted and the hatch rate was calculated (number of eggs produced divided by the number of eggs produced that hatched).

The egg hatch rates for OX4360 lines crossed to OX3069C are shown in Figures 5.23 and 5.24. A one-way ANOVA test showed that there are no significant differences in

the hatch rate between phenotypic groups in all OX4360 lines: OX4360A ($F_{4,45}$=1.063, p=0.386); OX4360B ($F_{4,45}$=0.665, p=0.619); OX3460C ($F_{4,45}$=1.153, p=0.344) and OX4360D ($F_{4,45}$=1.261, p=0.299).

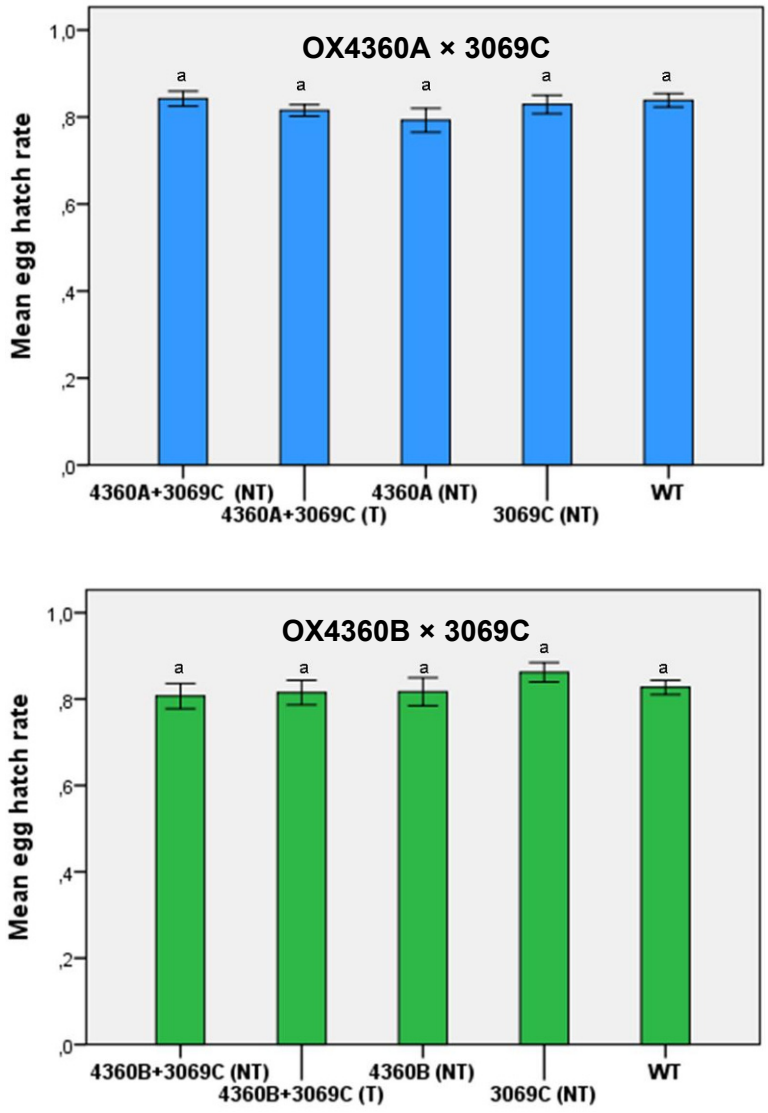

Figure 5.23. Hatch rate of eggs from females carrying one, both or neither OX4360A and B and OX3069C, reared off (NT) or on tetracycline (T). For all groups n=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

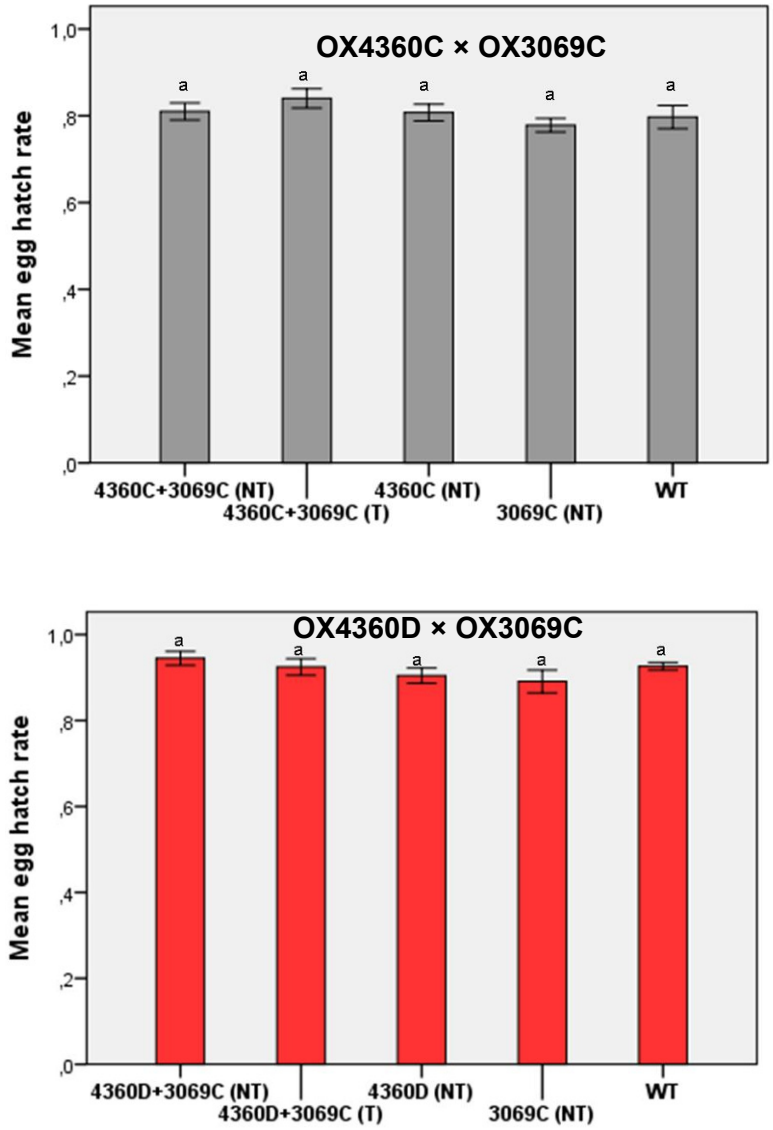

Figure 5.24. Hatch rate of eggs from females carrying one, both or neither OX4360C and D and OX3069C, reared off (NT) or on tetracycline (T). For all groups n=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

For both OX4498 lines, no significant differences in hatch rates were observed between phenotypic groups (Figure 5.25): OX4498A ($F_{4,45}$= 1.435, p=1.645); and OX4498B ($F_{4,45}$=1.488, p=2.22).

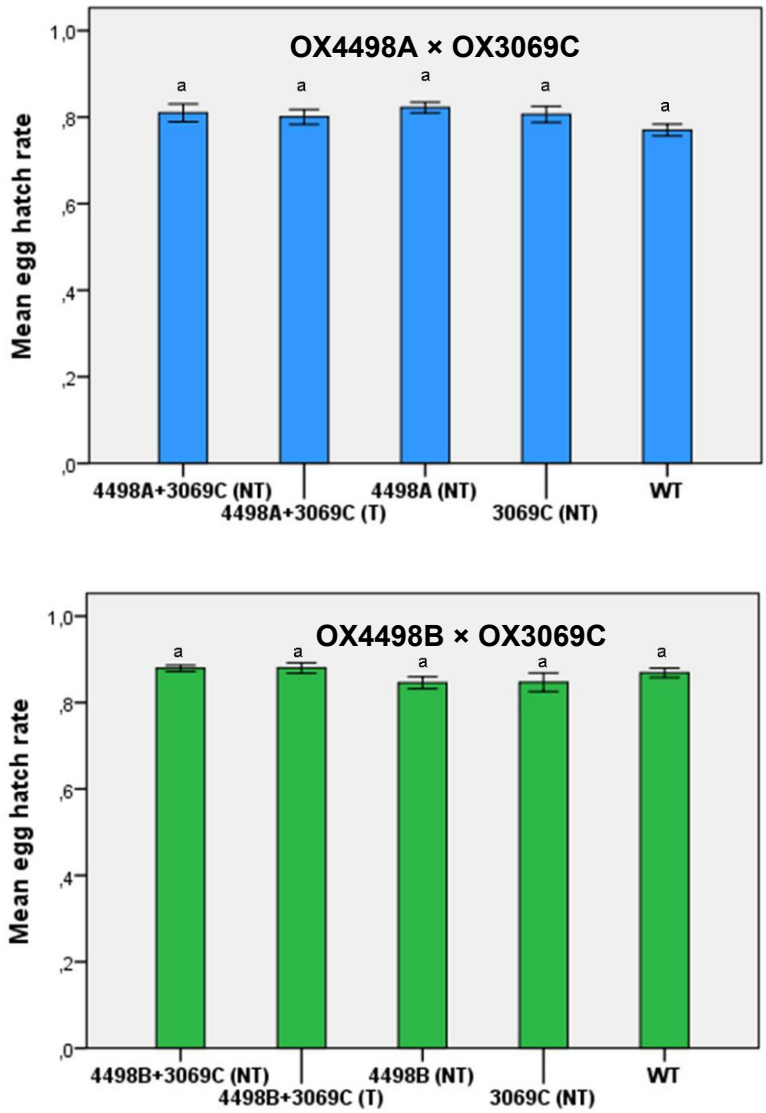

Figure 5.25. Hatch rate of eggs from females carrying one, both or neither OX4498A and B and OX3069C, reared off (NT) or on tetracycline (T). For all groups n=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

The egg hatch rates for the OX4521 driver lines crossed to OX3069C are shown in Figures 5.26, 5.27 and 5.28. A one-way ANOVA showed that there are no significant differences in the hatch rate between phenotypic groups in the following OX4521 lines: OX4521A ($F_{4,45}$=1.484, p=0.223), OX4521B ($F_{4,45}$=1.434, p=0.238), OX4521C ($F_{4,45}$=0.607, p=0.660), OX4521D ($F_{4,45}$=2.285, p=0.750), OX4521J ($F_{4,45}$=0.657 , p=0.625) and OX4521K ($F_{4,45}$=0.470, p=0.757). Significant differences occurred in

lines F and G. In OX4521F, the egg hatch rates for the double-hemizygous group reared off tetracycline was significant lower than the other groups ($F_{4,45}$=4.492, $p<0.05$, one-way ANOVA followed by a *post hoc* Dunnett's T3 test). In OX4521G, the egg hatch rate for wild-type was significantly lower than the other groups OX4521G ($F_{4,45}$=9.403 $p<0.05$, one-way ANOVA followed by a *post hoc* Dunnett's T3 test) (Figure 5.24).

As mentioned previously, we do not anticipate the transgene insertions to increase fecundity, this result is likely due to chance. I counted eggs laid by 10 females from each group, which might be considered an insufficient sample size and therefore a limitation of the experiment. However, my objective was to conduct preliminary assessments with these small samples and, in the event of finding large differences in fecundity or egg hatch in a certain group, to assess them in larger numbers. In this case, the small difference between wild-type females and the other groups did not merit further investigation.

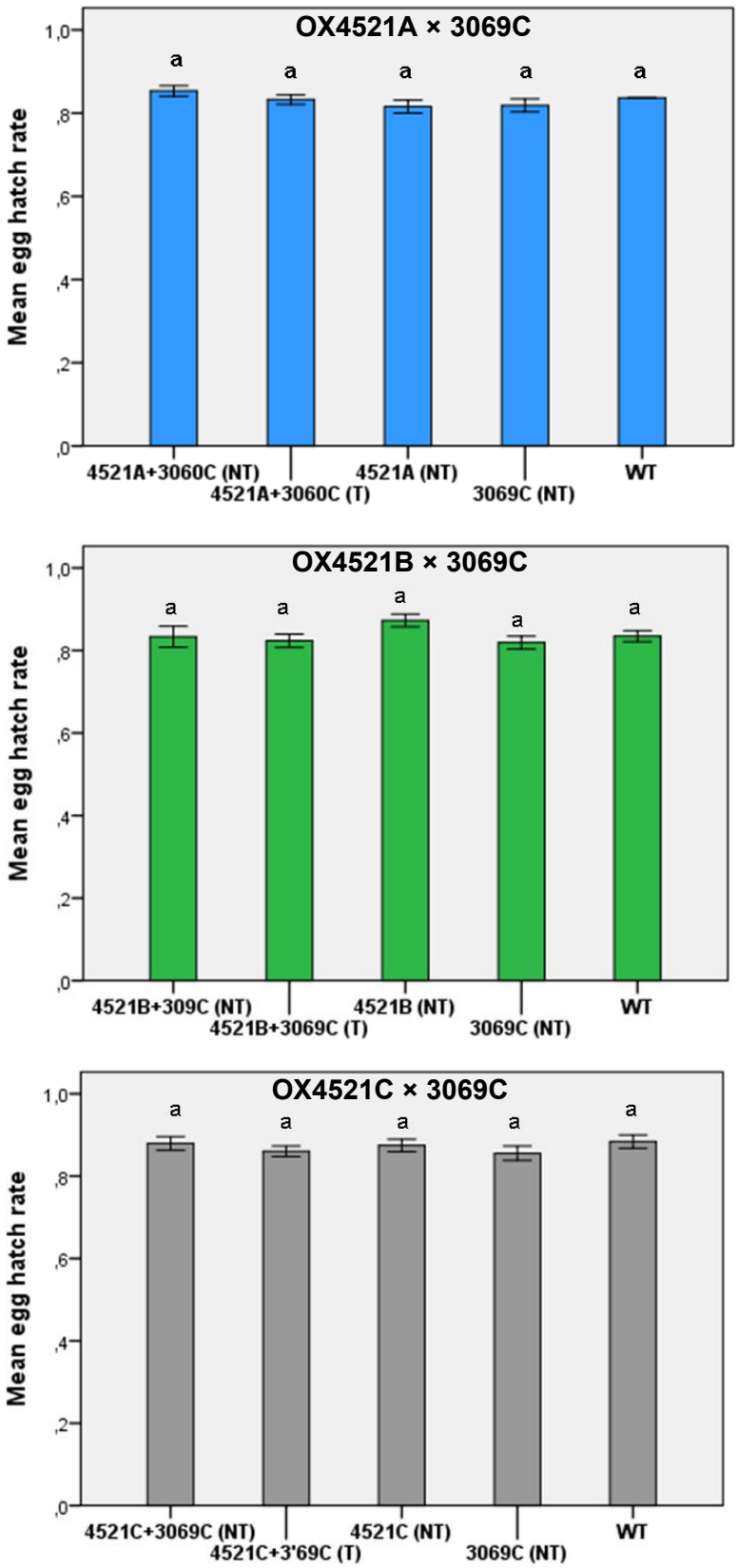

Figure 5.26. Hatch rate of eggs from females carrying one, both or neither OX4521A, B and C, and OX3069C, reared off (NT) or on tetracycline (T). For all groups n=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

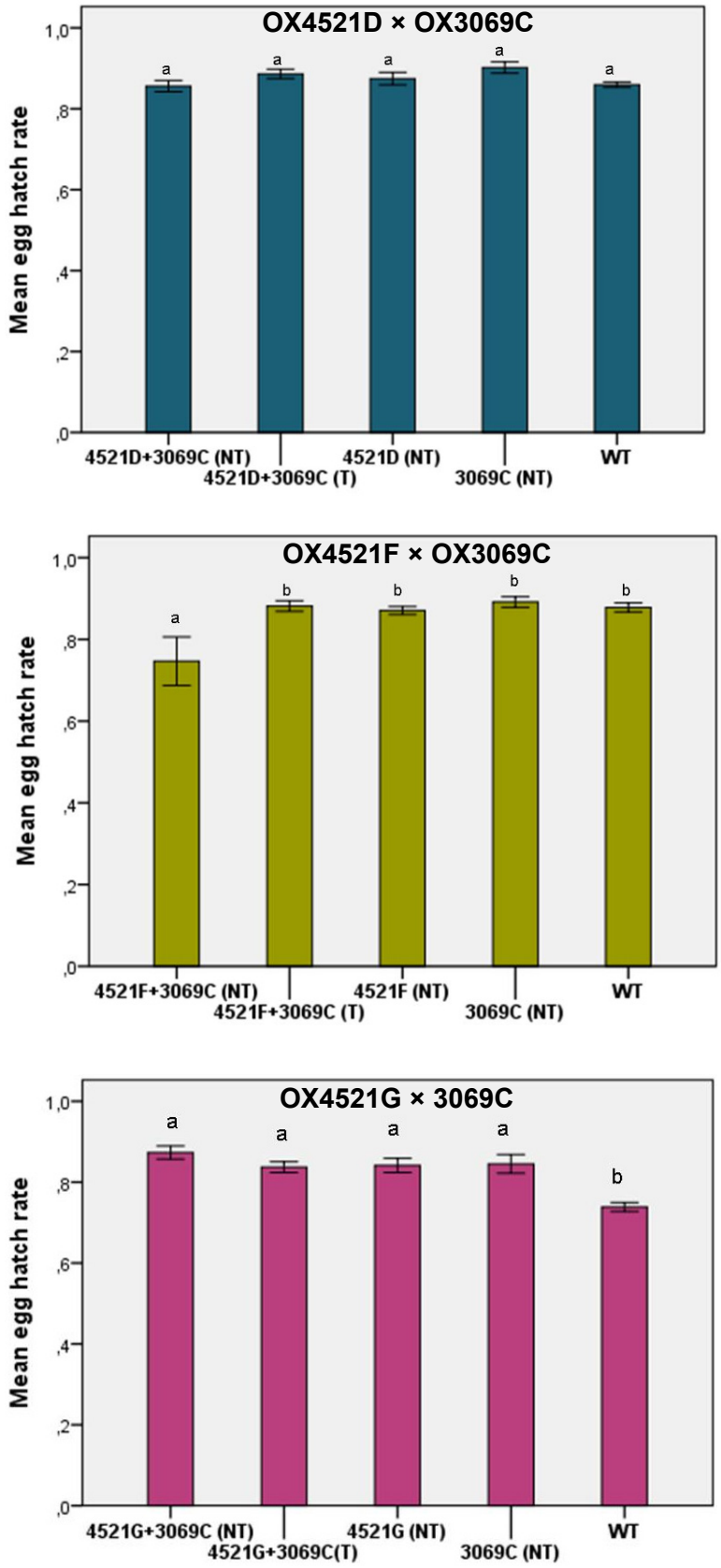

Figure 5.27. Hatch rate of eggs from females carrying one, both or neither OX4521D, F and G, and OX3069C, reared off (NT) or on tetracycline (T). For all groups n=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

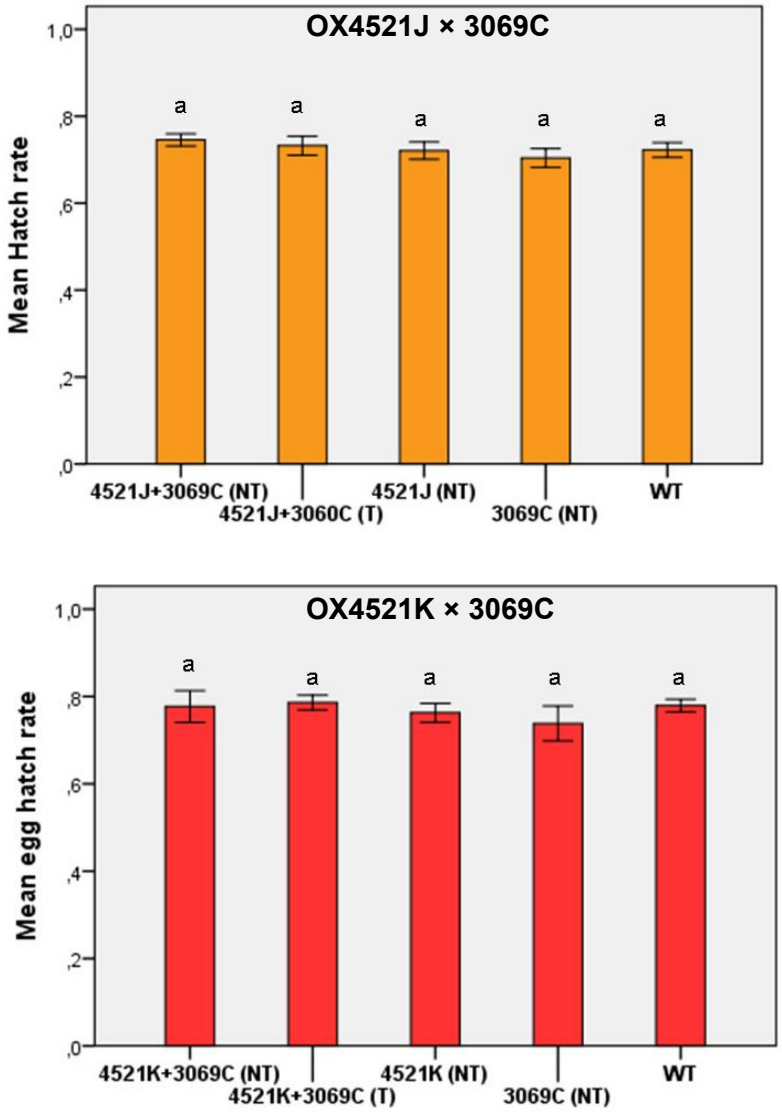

Figure 5.28. Hatch rate of eggs from females carrying one, both or neither OX4521J and K, and OX3069C, reared off (NT) or on tetracycline (T). For all groups n=10. Error bars represent the standard error of the mean. WT = wild-type reared on non-tetracycline diet. Groups marked with the same letter are not significantly different from each other.

In some lines, such as OX4360C, both OX4498 lines, OX4521A, B and J, there was a statistical significant reduction in egg production from double-hemizygous females, reared off tetracycline, compared to some or all the control groups. The decrease in egg production ranged, among lines, from 10% to 25%, except for line OX4521B where reduction reached a maximum of 38%. Although, these reductions were

statistically significant, they do not have a strong enough effect to be potentially useful.

In terms of egg hatch rates, from all driver lines tested only line OX4521F showed a decrease in egg hatch - approximately 15% - compared to the other groups. In practical terms for field release, in experiments with hemizygous individuals (carrying one copy of the RIDL transgene), such as the ones carried out here, we might view a 50% reduction in egg production to be approaching a useful phenotype (Luke Alphey personnal communication). It may be that the extra transgene copy in homozygous individuals would further reduce egg production. Therefore, as mentioned above for OX4360C, both OX4498 lines, OX4521A, B and J, although a 15% reduction in egg hatch is statistically significant, it does not have a strong enough effect to be useful, in practical terms.

In lines OX4360, OX4498 and OX4521D and F, where no AmCyan expression was observed nor a decrease of >50% in egg production or hatch rate; it is possible that tTAV is not being expressed or it is not being expressed in sufficient quantities to drive the expression of AmCyan and *reaper*^{KR}.

In lines OX4521C and G, AmCyan expression was observed with the reporter line (showing that tTAV is being expressed), but a significant decrease in egg production or egg hatch was not observed in effector line crosses. It is be possible that tTAV is binding to *tetO* sequence but the consequent enhancing effect is insufficient to drive lethal expression levels of *reaper*^{KR}.

In lines OX4521A, B, J, and K, the AmCyan reporter was expressed and there was a significant, tetracycline-repressible, decrease in egg production in the effector line crosses. It is possible that tTAV is driving the expression of *reaper*^{KR}, causing some

lethality. However, *reaper*^{KR} expression might not be strong enough or at the correct time to cause more severe lethality.

5.3.7. Reverse-transcription PCR for tTAV expression

Reverse-transcription PCR (RT-PCR) was used to detect tTAV expression in female ovaries of all OX4360 and OX4498 lines, and OX4521D and F lines. AmCyan expression nor a decrease of >50% in egg production of hatch rate was observed in these line, an indication that tTAV might not being expressed in these lines or at least not at sufficient quantities to cause an effect. RT-PCR was performed on ovaries and respective carcasses of double-hemizygous females, reared on and off tetracycline. Male controls reared on and off tetracycline, were included to test sex-specificity of tTAV expression.

RT-PCR results show that the expected tTAV fragment of 261 bp was not amplified from the ovaries or carcasses cDNAs for all OX4360 (Figure 5.29) and OX4498 lines (Figure 5.30) crossed to OX3069C effector strain. This indicates that in these lines, as indicated by the lack of AmCyan expression, tTAV is not being expressed, or at least not being expressed at detectable levels.

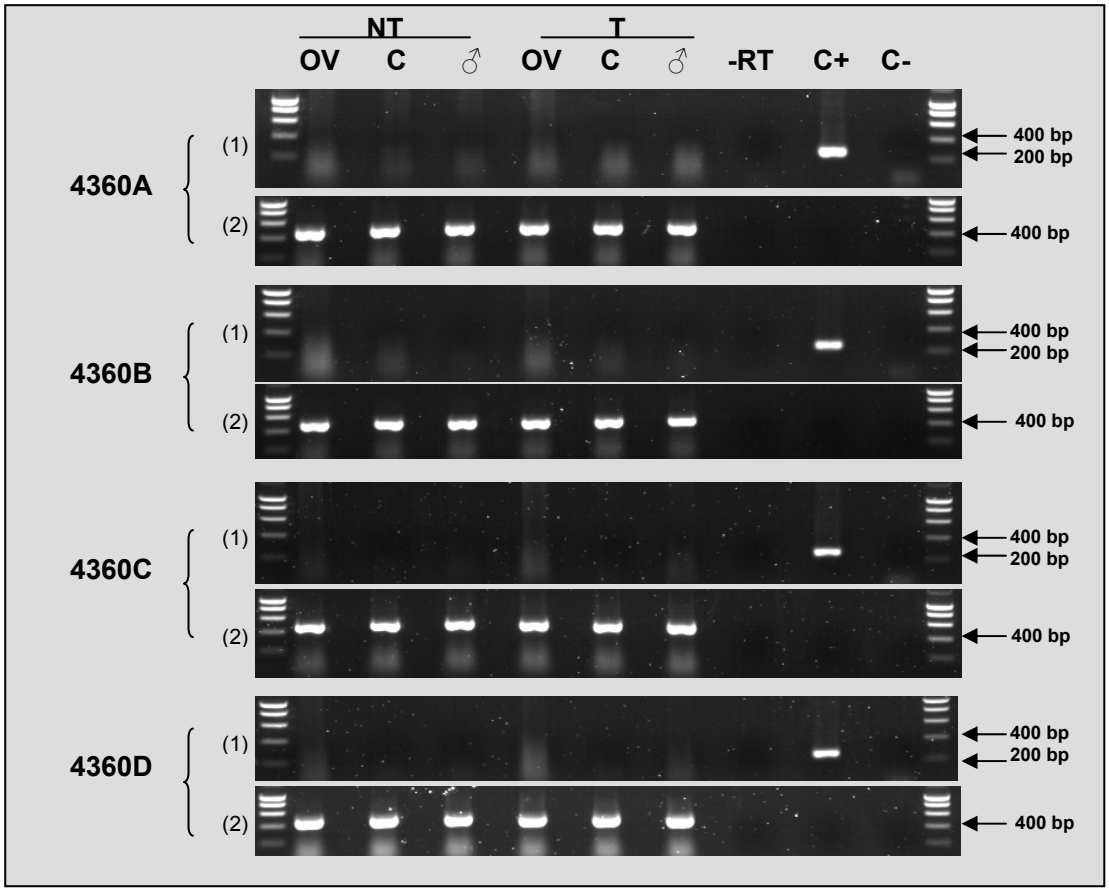

Figure 5.29. tTAV transcription in OX4360 lines. (1) Transcripts were amplified with primers 852 and 853. Expected amplicon size for cDNA was 261 bp. Samples included: Ov, female ovary; C, female carcass; ♂, male; all reared off (NT) and on tetracycline (T); -RT, no reverse transcriptase negative control; C+, OX4360 plasmid positive control; and C-, no-cDNA negative control. (2) A PINK BOLLWORM *heat shock protein 83 (hps83*) control, amplified with primers 2628 and 2629 with an expected amplicon size of 477 bp, was included. Males reared off and on tetracycline, and ovary and carcass from females reared on tetracycline, were included as controls. Smart ladder is run at both ends.

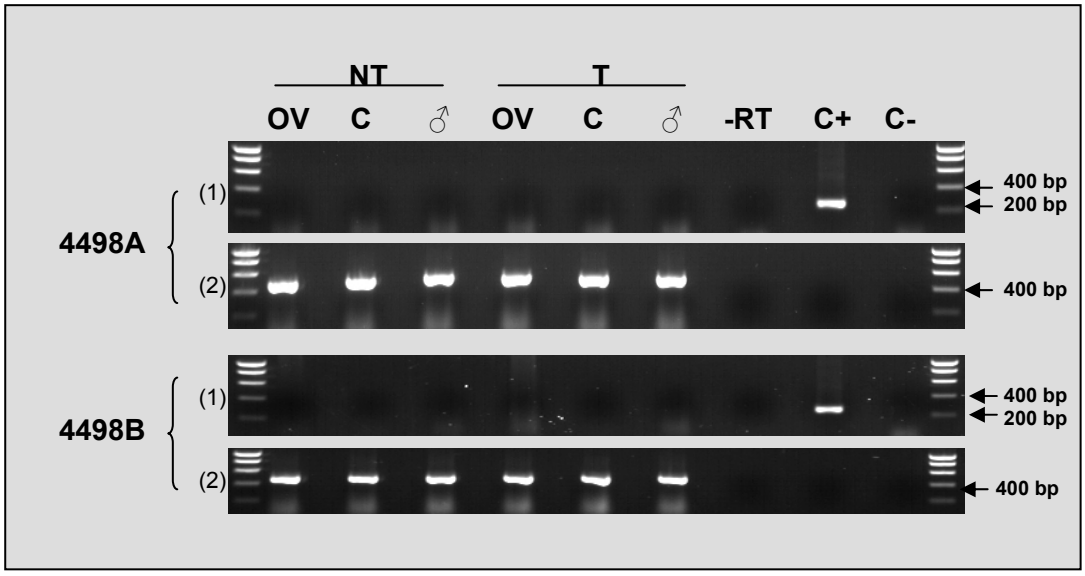

Figure 5.30. Detecting transcription of tTAV in OX4498 lines. (1) Transcripts were amplified with primers 852 and 853. Expected amplicon size for cDNA was 261 bp. Samples included: Ov, female ovary; C, female carcass; ♂, male; all reared off (NT) and on tetracycline (T); -RT, no reverse transcriptase negative control; C+, OX4498 plasmid positive control; and C-, no-cDNA negative control. (2) A PINK BOLLWORM *heat shock protein 83 (hps83*) control, amplified with primers 2628 and 2629 and an expected amplicon size of 477 bp, was included. Males reared off and on tetracycline, and ovary and carcass from females reared on tetracycline, were included as controls. Smart ladder is run at both ends.

For OX4521D, RT-PCR results also show that tTAV is not being expressed, or at least at detectable levels, as cDNAs did not yield the expected 261-bp amplified fragment. For OX4521F, a very faint 261-bp PCR product was produced. As this line did not drive expression of AmCyan, when crossed to the *tetO*-AmCyan reported line, it indicates that the very low levels of tTAV being expressed in this line are not sufficient to drive AmCyan expression (Figure 5.31).

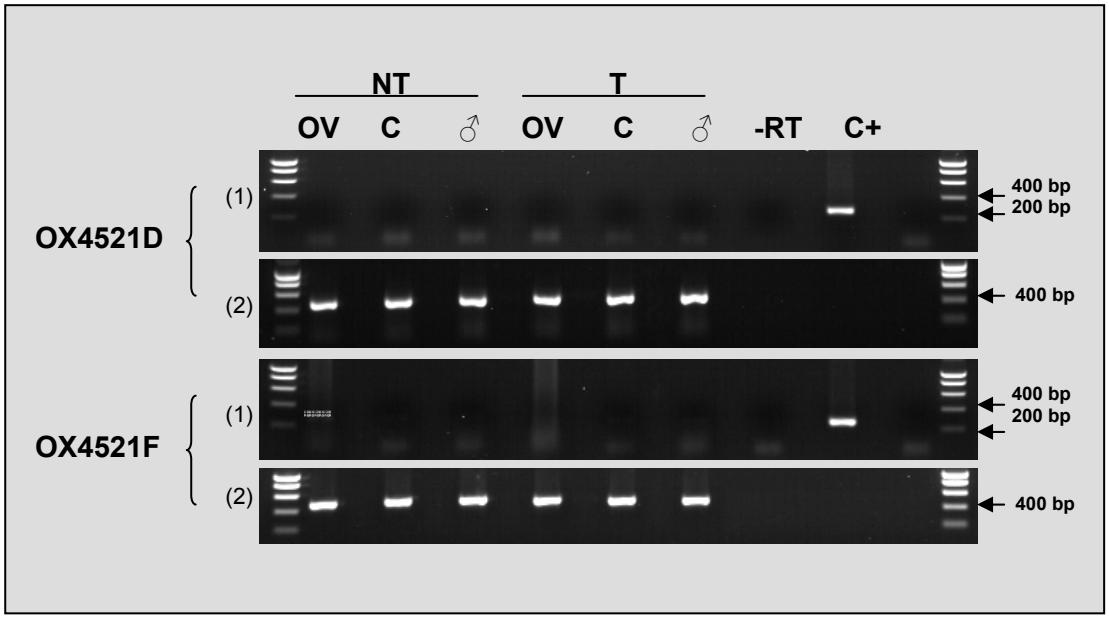

Figure 5.31. Detecting transcription of tTAV in OX4521 lines. (1) Transcripts were amplified with primers 852 and 853. Expected amplicon size for cDNA was 261 bp. Samples included: Ov, female ovary; C, female carcass; ♂, male; all reared off (NT) and on tetracycline (T); -RT, no reverse transcriptase negative control; C+, OX4498 plasmid positive control; and C-, no-cDNA negative control. (2) A PINK BOLLWORM *heat shock protein 83 (hps83*) control, amplified with primers 2628 and 2629 and an expected amplicon size of 477 bp, was included. Males reared off and on tetracycline, and ovary and carcass from females reared on tetracycline, were included as controls. Smart ladder is run at both ends.

5.3.8. Reverse-transcription PCR for *reaper*^{KR} expression

To investigate whether *reaper*^{KR} was being expressed in the OX4521A, B, C, F, G, J and K lines, RT-PCR was performed on ovaries and respective carcasses of double-hemizygous females, reared on and off tetracycline. Male controls, reared on and off tetracycline, were included to test sex specificity of *reaper*^{KR} expression.

RT-PCR show that the 195-bp expected product was not amplified from any of the cDNA samples analysed, except in the OX4521 B and C lines (Figure 5.32). Therefore, *reaper*^{KR} is not being expressed in the ovaries of females reared off tetracycline or in any other sample included, at least at detectable levels, in any of the OX4521A, F, G, J and K lines.

RT-PCR results show that the 195-bp expected product was amplified from ovary cDNA of females, reared off tetracycline, in lines OX4521B and C, but not in the female carcass and male cDNA samples. This confirms that expression of *reaper*^{KR} was sex- and tissue-specific. As expected product was amplified in samples from individuals reared off tetracycline and not in samples of individuals reared on tetracycline, results show that the system is tetracycline-repressible, in these lines.

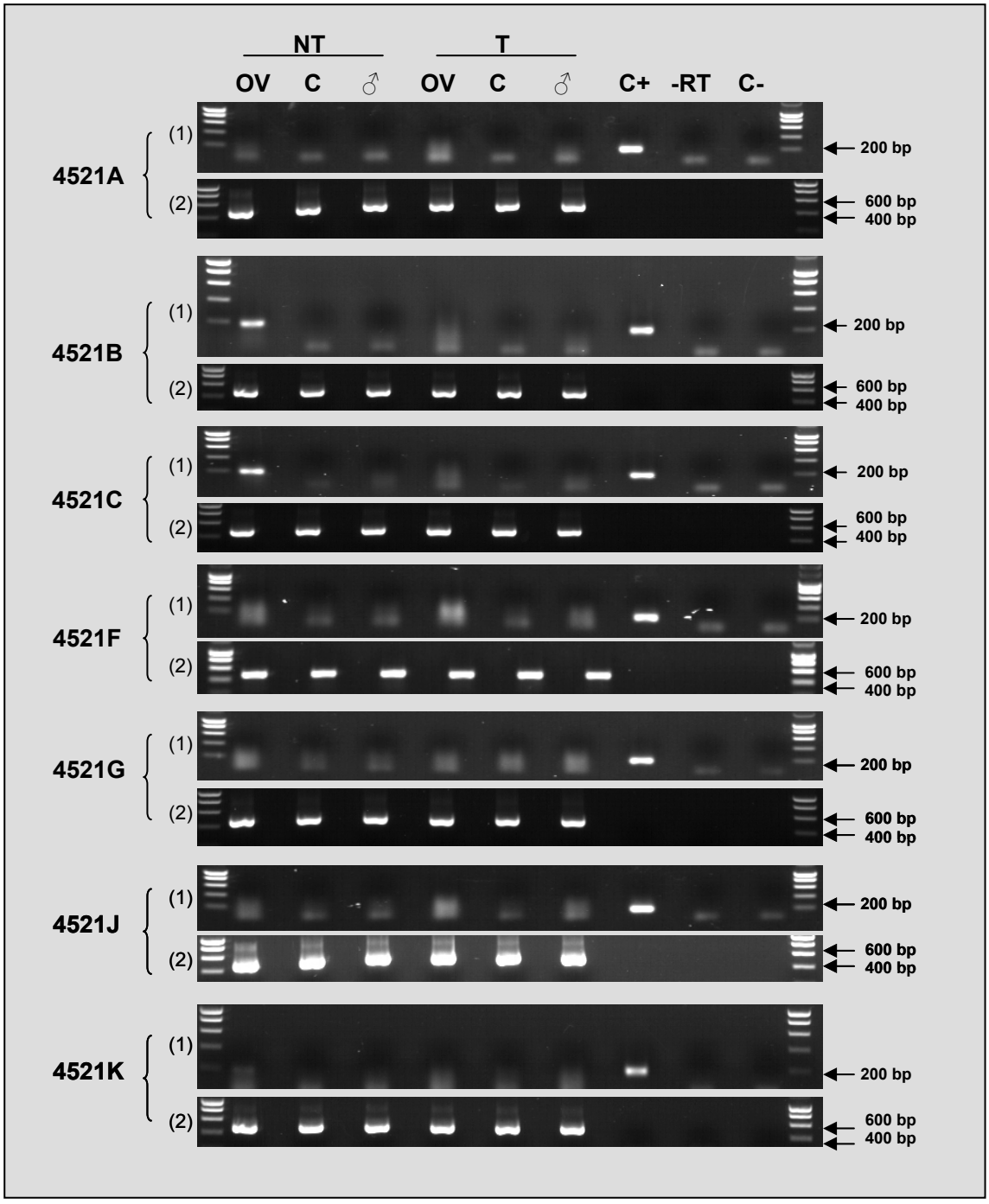

Figure 5.32. Expression of *reaper*^{KR} in OX4521 lines. (1) Transcripts were amplified with primers 963 and 964. Expected sizes for cDNA was 195 bp. Samples included: Ov, female ovary; C, female carcass; ♂ - male, all reared off (NT) and on tetracycline (T); -RT , no reverse transcriptase negative control; C+, OX4360 plasmid positive control; and C-, no-cDNA negative control. (2) A PINK BOLLWORM *heat shock protein 83 (hps83*) control, amplified with primers 2628 and 2629 and an expected and size of 477 bp, was included (lower panels for each line). Males reared off and on tetracycline, and ovary and carcass from females reared on tetracycline, were included as controls. Smart ladder is run at both ends.

5.4. Discussion

Three putative promoter regions from *B. mori* genes - chorion peroxidase, egg-specific protein and vitelline membrane protein 30 - were shown to be active in pink bollworm, driving detectable expression levels of the ZsGreen reporter gene exclusively in the female ovaries (Table 5.6). Though precise correspondence of expression pattern with *B. mori* was not assessed, this suggests that the sex and tissue specificity of these promoters is functionally conserved when inserted into the pink bollworm genome. ZsGreen expression induced by the chorion peroxidase and VMP30 putative promoters was limited to almost fully mature follicles. The ESP putative promoter induced ZsGreen expression in most stages of follicle development.

Table 5.6. Summary data for ZsGreen ovary-specific expression in pink bollworm lines transformed with OX4270, OX4413 and OX4660.

Construct	*B. mori* putative promoter	No. transgenic lines generated	No. transgenic lines examined for ZsGreen expression	No. transgenic lines expressing ZsGreen
OX4270	Chorion peroxidase (BmChPxd)	19	9	3
OX4413	Egg specific protein (ESP)	7	7	4
OX4660	Vitelline membrane protein 30 (VMP30)	4	4	4

Test crosses with the chorion peroxidase (OX4360) and the VMP30 (OX4498) putative promoter indicated that these sequences were not able to drive adequate expression of the optimised tetracycline-controlled transactivator (tTAV). When hemizygous OX4360 (chorion peroxidase-tTAV) or OX4498 (VMP30-tTAV) were crossed with hemizygous OX4026 (*tetO*-AmCyan) reporter lines, no AmCyan expression was detected in the ovaries of females reared on or off tetracycline. Similar

results were obtained when the same lines were crossed to OX3069C strain (*tetO-reaper*^{KR}). Expression of tTAV was anticipated to induce expression of the *reaper*^{KR}, resulting in death of the ovary follicle cells, and consequent reduced fecundity. Analysis of the egg production and hatch rate, in OX4360 and OX4498 lines, indicates that there was no significant reduction in fecundity in double hemizygous females reared off tetracycline compared with double hemizygous females reared on tetracycline (Table 5.7), except in line OX4360C where a statistically significant decrease, but not a strong enough effect to be useful, in egg production (14.1%) was observed in females reared on tetracycline compared to females reared off tetracycline. Further analysis by RT-PCR indicated that tTAV is not being produced, at least at detectable levels, in any of these lines. Therefore, the decrease in egg production observed in line OX4369C is unlikely to be due to tTAV driving *reaper*^{KR} lethality, causing a decrease in fecundity. It appeared that the chorion peroxidase and the VMP30 putative promoters drive expression of insufficient levels of tTAV to induce cell death, in these lines context. It is also possible that an insufficient number of lines were tested, four and two lines for OX4360 and OX4498, respectively. Generation of more lines to account for position effects might be needed.

The chorion peroxidase and BmVMP30 gene expression was to be limited to nearly-mature follicles (see Chapter 5, section 5.3.3). Such a limited expression window might indicate that the putative promoters used might act insufficiently to induce tTAV expression at sufficient levels to drive AmCyan and *reaper*^{KR} expression.

Table 5.7. Summary of the data obtained from test crosses to OX4026D and OX3069C. The change, increase (↑) or decrease (↓), in egg production and hatch rate between double-hemizygous females reared on and off tetracycline is expressed as a percentage of results obtained from females reared on tetracycline (Tet).

Line		Putative promoter	AmCyan expression	Difference between females reared on/off Tet	
				Egg production (%)	Hatch rate (%)
OX4360	A	BmChPxd	×	↓ 6.3	↑ 3.6
	B		×	↑ 0.8	↓ 1.4
	C		×	↓ 14.1*	↓ 3.6
	D		×	↑ 0.8	↑ 2.2
OX4498	A	BmVMP30	×	↑ 0.8	↑ 1.2
	B		×	↓ 0.2	↓ 0.1
OX4521	A	ESP	✓	↓ 14.5*	↑ 2.5
	B		✓	↓ 29.0*	↑ 1.2
	C		✓	↑ 8.4	↑ 2.2
	D		×	↓ 2.1	↓ 3.5
	F		×	= 0.0	↓ 15.3*
	G		✓	↓ 2.8	↑ 4.3
	J		✓	↓ 24.6*	↑ 1.8
	K		✓	↓ 9.5	↓ 1.2

*The difference between on- and off-tetracycline results was found to be statistically significant.

Test crosses with hemizygous OX4521A, B, C, G, J and K lines (ESP-tTAV) and the reporter line OX4026D (*tetO*-AmCyan) showed that the egg-specific protein putative promoter could drive expression of tTAV and, as a result, expression of the AmCyan marker (Table 5.7). AmCyan expression appeared to be fully repressible by tetracycline, as equivalent insects reared on a diet containing tetracycline showed no such fluorescence. However, in OX4521D and F, AmCyan was not detected in the ovaries. RT-PCR showed that tTAV was not being expressed in line OX4521D and was being expressed at very low levels in OX4521F. It is possible that in the OX4521D and F lines, the transgene is inserted in an area of the genome of low

transcriptional activity or under the influence of silencing DNA sequences, resulting in undetectable or very low levels of tTAV expression.

Test crosses with OX4521 lines and OX3069C (*tetO*-reaper^{KR}) showed a statistically significant effect on fecundity in the absence of tetracycline (Table 5.7) for several OX4521 lines (OX4521A, B, F and J). Further analysis by RT-PCR showed that in lines OX4521A, G, F, J and K, *reaper*^{KR} transcripts were not detectable with these PCR conditions in the ovaries of females from these lines, suggesting that tTAV might be binding to *tetO* sequence but that the consequent enhancing effect is insufficient to drive lethal expression levels of *reaper*^{KR}. Therefore, any significant decrease in egg production or egg hatch in lines OX4521A, F and J is unlikely to be a result of expression of *reaper*^{KR}. It might be due to expression of tTAV, which can be toxic to cells. There is also the possibility of accidental tetracycline contamination in these lines.

In OX4521B and OX4521C, *reaper*^{KR} expression was detectable and limited to the ovary of females reared off tetracycline, indicating that *reaper*^{KR} expression is repressible by tetracycline in these lines as *reaper*^{KR} expression was not detected in ovaries of females reared on tetracycline. In the OX4521B and C lines, where tTAV and *reaper*^{KR} have been shown to be expressed, a statistically significant decrease in egg production between 29% and 39% was observed compared to the control groups were observed in line OX4521B but not in line OX4521C. Differences in lethality levels might be due to different in insertion sites and its position effects. The low lethal effect in these lines might indicate that the time of *reaper*^{KR} expression may be too late or at insufficient levels to cause significant damage to the follicle cells. Furthermore, RT-PCR analyses performed in this study was not quantitative, so if there was a fine threshold in expression levels between causing an effect, and not,

then these would possibly not detect those differences. Accidental tetracycline contamination cannot be disregarded. If OX4521 and OX3069C parents and/or their progeny were in contact with tetracycline, even at low doses, it might have been sufficient to repress expression of *reaper*^{KR}.

In this study, lethality, and its subsequent effect on fecundity, was assessed in trans-hemizygous insects since – with field application in mind – this must be sufficient to induce significant functional sterility. In terms of potential for use in a control programme, we anticipate that a survival rate of a RIDL line (here egg production / egg hatch), off tetracycline, should be <10%. Are the molecular components analysed in this study likely to produce such high-penetrance sterility? All strains tested were hemizygous; one would anticipate that the extra transgene copy in homozygous individuals, the ones that would be released in the field, would further reduce egg production. Reductions of 29%, as observed in off-tetracycline egg production in the OX4521B, are a relatively modest step towards a target of >90%. Therefore, such reductions are statistically significant but not a strong enough effect to be useful. However, there may be several ways to improve this. It is difficult to 'improve' promoters by rational design as we know relatively little about structure-function relationships in non-coding elements. However, all other aspects can potentially be improved – we could further optimise the expression of tTAV and the effector, test additional effectors to find more potent ones. Finally, a rather simple step for additional expression might simply to put more than one copy of the system in the construct (suitably engineered to avoid recombination-mediated instability).

It is not clear if the decrease in egg production in line OX4521B is due to *reaper*^{KR}, shown to be expressed by RT-PCR, and its negative effect on egg production or if it is due to chance. In this line, a statistical significant decrease was observed in off-

tetracycline egg production compared on-tetracycline egg production and other controls, indicating that it might be affecting the follicle cells and, thus, egg production. However in line OX4521J, for instance, there was also observed a similar decrease in egg production (24.6%) but most likely to be due to chance as RT-PCR analysis showed *reaper*^{KR} is not being expressed in this line. Likewise, the decrease observed in OX4521B could also by due to chance. Perhaps the sample size used, 10 females per group, is not representative and larger sample sizes should be considered in future work. Alternatively, it is possible that *reaper*^{KR} simply does not induce apoptosis at the levels seen, or that the follicle cells targeted do not play as an important role in embryo formation as initial thought. It is also possible that small changes in chorion quality did not affect survival in laboratory conditions but would have a greater impact in the field due to unpredictable and more severe environmental conditions.

Nevertheless, assuming that a degree of tetracycline-repressible reduction in egg production was observed in OX4521B (29%), further improvements could be made to reduce fecundity. For instance, in order to increase off-tetracycline cell mortality (and thereby fecundity), the copy number of the *tetO* sequence could be increased from seven copies to 21. This would provide more binding sites for the tTAV protein, increasing expression of the effector protein. Another alternative to improve the system would the inclusion of another pro-apoptotic gene, also under the control of the *tetO* sequence (Zhou et al., 1997; Wing et a., 1998). However, new lethal genes would need to be characterised and/or tested in pink bollworm.

Chapter 6

Summary and conclusions

6.1. Summary

This thesis focuses on the development of methods and components for moth genetic engineering, laying the groundwork for the future development of genetic control systems. Successful transformation of the diamondback moth was achieved using the *piggyBac* transposable element (Chapter 3). Site-specific recombination using the ΦC31 integrase and the Cre and Flp recombinases have also been successfully used in the manipulation of the diamondback moth genome (Chapter 4). Potential use of *B. mori* regulatory elements in future development of female sterile pink bollworm strains was also investigated (Chapter 5).

6.2. Diamondback moth transgenesis

Successful transformation of the diamondback moth was achieved using the *piggyBac* transposable element (Chapter 3); no prior instance of successful transformation of this species is known. Minimum transformation efficiency ranged from 0.48% to 0.68%. Subsequently, a maximum of 2.5% efficiency was achieved, possibly reflecting improved technique. Nevertheless, relatively low efficiencies were obtained when compared to other published efficiencies in Lepidoptera (Peloquin et al., 2000; Tamura et al., 2000). It is possible that low transformation is a result of the use of *piggyBac*. Studies carried out in *B. mori* and *T. ni* (the *piggyBac* host of origin) suggest that the presence of endogenous *piggyBac* or *piggyBac*-like elements already present in the insects might hamper the movement of the transgene (Zhong et al.,

2007; Lobo et al., 1999), possibly by influencing excision and transposition of the transgene (Lobo et al., 1999). A similar phenomenon was observed in *Drosophila*, where movement of the *P*-element was hampered in strains already carrying the transposon (Rio et al., 1990). *piggyBac*-like elements have been found in some lepidopteran species such as *Helicoverpa armigera*, *Helicoverpa zea* and *Spodoptera frugiperda* (Zimowska & Handler, 2006; Wang et al., 2010) with 78% or greater nucleotide sequences similarity to the canonical *piggyBac* derived from *T. ni*. It is possible that such elements are also present in the diamondback moth and affecting transformation efficiency. Alternatively, transposase expression levels could be improved if the *Drosophila hsp70* promoter used to drive the transposase in our helper plasmid were replaced by a promoter from a lepidopteran homologue.

In this study it was not possible to determine the exact transformation rates as pooled injection survivors were crossed in pools rather than individually. Therefore, transformation rates should be seen as minimum estimates. Transformation efficiencies could potentially be improved by determining precisely where the pole cells (which develop into germline cells) are located in the preblastoderm embryo. In this study, microinjections were carried out within one hour of egg laying. It would also be useful to determine the age of the embryos at which cellularisation begins, so that injection can be carried out at pre-blastoderm stage. Nevertheless, the efficiency obtained enables regular transformation of diamondback moth.

6.3. Promoter activity in diamondback moth

Appropriate promoters to drive the expression of marker genes are an important component of successful transformation systems. Three promoters, Hr5*ie1*, *Opie2* and

3×P3, selected according to their history of previous use for transformation of other insect species, were shown to be transcriptionally active in diamondback moth.

Both *Opie2* and Hr5*ie1* drove the expression of a fluorescent marker in many tissues, through most life stages, except early embryos. The 3×P3 promoter also drove the expression of a fluorescent marker throughout most developmental stages, except embryos. However, marker expression driven by 3×P3 was not as easily detected at late pupal stages as that regulated by *Opie2* and Hr5*ie1*.

It is anticipated that promoters isolated from other lepidopteran species may also show high activity in diamondback moth, such as the *B. mori* cytoplasmic actin gene *BmA3* promoter, which has been successfully used in the transformation of pink bollworm and codling moth (Peloquin et al., 2000; Ferguson et al., 2010).

6.4. Marker genes for diamondback moth transformation

The suitability of two fluorescent marker proteins – DsRed2 and ZsGreen – as genetic markers in diamondback moth was demonstrated. Both markers were clearly visible and easily screened at different life stages, including adult stage. This is a particularly important feature; it not only allows easy separation of transgenic from non-transgenic individuals in the laboratory, but also but is also useful in a RIDL setting. Released insects must be marked in order for relative numbers of released and wild insects can be monitored in the field, and these fluorescent protein markers show promise for such a purpose.

The ability to use different markers is particularly useful in tissue-specific expression assays where a transformation marker and a tissue-specific reporter marker are needed. It is also useful when two transgenic strains are crossed together to test

combinations of transgene components. Other fluorescent genes have been recently identified such as the "mFruits" (Day & Davidson, 2009). If such genes could be used solely or in addition to the above DsRed2 and ZsGreen markers it would be a useful tool in diamondback moth transgenesis.

6.5. ΦC31-mediated site-specific integration in diamondback moth germline

Site-specific genomic integration was attained using the ΦC31 integrase when the docking sites and the incoming plasmids contained *attP* and *attB* recognition sites, respectively. The estimated integration efficiency was between 0.16% and 0.39%, depending on the recipient line genomic position. Efficiencies obtained were lower than the reported 23% by Nimmo et al. (2006) and 1.24% efficiency by Labbé et al. (2010b) in mosquitoes, using recombination sites of the same as in this study and the same source of recombinase. These differences might be due to species-specific factors interfering with recombination.

Results suggest that integration efficiency seems to depend on the recipient line genomic location, as previously observed in *Drosophila* (Bischof et al., 2007) and in mouse embryonic stem cells (Monetti et al., 2011). Position effects and secondary structure of the genomic DNA surrounding the transgene might restrict access to the *attP* site by the recombinase. Relative position of the *attP* site and other construct elements were also shown to strongly influence recombination efficiency. The presence of certain DNA sequences, such as Hr5*ie1* and SV40 poly(A), adjacent to the *attP* site seemed to inhibit recombination. It is possible that repeats or other

structural elements within these sequences interfere with the homology search by the recombinase, making recognition of the site difficult. In addition, the secondary structure of these repetitive elements might impair the recombinase's access to the recognition site and/or inhibit formation of the synaptic complex. In this study, recombination reactions were successful when the *attP* site was separated, by approximately 300 bp, from other repetitive elements in the construct. Although it is difficult to precisely pinpoint the minimum desirable distance, as no further investigations to determine it were carried out, it is recommended that, for design of future such constructs, a distance not less than 300bp between the *attP* and other elements of the construct should be maintained.

It would be interesting to test whether smaller *attP* and *attB* sequences are functional in diamondback moth. *attP* and *attB* sequences ranging from 39 bp to 300 bp and 34 bp to 285 bp, respectively, have been used with success in several systems, such as humans cells, *E. coli*, medfly and *Drosophila* (Groth et al., 2000; Bateman et al., 2006, Thorpe & Smith, 1998; Schetelig et al., 2009; Gao et al., 2008; Bischof et al., 2010). In my study, a 221-bp *attP* site and a 285-bp *attB* site were used but it would be advantageous to use smaller sites, especially the *attP* site present in the recipient construct inserted by *piggyBac*, as transformation efficiencies with transposable elements decreases with transgene size (Geurts et al., 2003; Lorenzen et al., 2003).

The lack of detectable activity of optimised integrases in catalysing ΦC31-mediated integration was unexpected. The low survival rates (10 and 13%) obtained when injecting the optimised integrase source without the nuclear localisation signal (NLS) attached to its N- and C-termini, might indicate that the codon optimisation has led to more efficient translation, resulting in toxic build-up of protein. ΦC31-mediated recombination is not fully specific to its cognate sites. Pseudo-sites are native

genomic sequences that are related to *attP* or *attB*, and serve as substrate for the activity of the integrase (Brown et al., 2010). Pseudo-integration events have been reported, where the donor construct is inserted in pseudo-sites (Nimmo et al., 2006). It is possible that the combination of pseudo-sites present in the diamondback moth genome and greater amounts of enzyme available in the cell nucleus might lead to recombination occurring at frequencies that are deleterious to the insect. The pseudo-integrations reported involved integration of the injected *attB* construct, as these have phenotypic consequences, however it is possible that optimised integrases may catalyse intra-genomic rearrangements. However, during the course of these experiments, pseudo-site integrations were not observed.

The presence of a NLS, at one or both ends of the integrase, seems to be of particular relevance for the efficiency of the ΦC31 integrase, as its size (67 kDa) does not allow the protein to enter the nucleus easily by passive diffusion (Andreas et al., 2002; Brown et al., 2010). However, the addition of a NLS flanking the optimised integrase did not result in recombination. The position of the NLS in the protein was shown to be critical in mammalian cells; addition of an NLS to the C-terminal of the protein increases efficiency whereas addition to the N-terminal can be detrimental. Addition of amino acids at the N-terminal likely interferes with its active centre (Andreas et al., 2002). Due to time constraints this possibility was not tested. However, for future work the NLS should be retained. An alternative would be the addition of a C-terminal NLS to the non-optimised protein, which has been reported to increase integration efficiencies in *Drosophila* and mammalian cells (Bischof et al., 2007; Andreas et al., 2002). As a result, the enzyme may still be directed to the nucleus but without being overproduced, leading to higher efficiencies without having a negative impact on the insect.

Nevertheless, even at lower transformation efficiencies, unidirectional site-specific integration mediated by the ΦC31 integrase is an attractive method since it eliminates much of the labour expended in characterising random transposon insertions. Furthermore, after identifying and locus at which insertion does not cause a significant fitness cost to the insect, transgenes can be repeatedly inserted into this suitable docking site. Secondary modification of the docking site will also allow the comparative functional analysis of different sequences (e.g. mutated resistance genes) without the interference of position effect.

6.6. Cre- and Flp-mediated excision of DNA segments in the diamondback moth germline

Cre- and Flp-mediated excision of DNA segments was shown to be highly efficient in diamondback moth, reaching 100% excision in both cases. Efficiencies achieved here are comparable to those seen in *Drosophila* and *Ae. aegypti* (Golic & Lindquist, 1989; Siegal & Hartl, 1996; Jasinskiene *et al.*, 2003).

The successful combinatorial use of the ΦC31 integrase and the Cre or Flp recombinases is a particularly useful tool in diamondback moth genome manipulation, in view of future field release of transgenic diamondback moth control programmes. As discussed in Chapter 4, it will allow the removal of plasmid origin of replication and antibiotic resistance sequences inserted into the insect genome by ΦC31-mediated integration. This is particularly useful in regulatory terms, due to concerns about theoretical horizontal gene transfer between species.

6.7. Recombinase mediated cassette exchange

All attempts to achieve recombinase-mediated cassette exchange (RMCE) in diamondback moth failed. RMCE combining the Cre and Flp pathways instead of heterospecific sites has been shown to work in embryonic stem cells (Lauth et al., 2002) but has never been attempted in insects. As previously mentioned (Chapter 4), one of the practical limitations of this experiment was the concentration of recombinase used. As Cre and Flp recombinase mRNA had to be co-injected, lower concentrations of recombinase capped mRNA were used: half of the concentration used in the excision assays. This and the problems associated with the quality and stability of the capped mRNA might have meant that insufficient recombinase mRNA was injected into the embryos. An alternative approach would be to replace the mRNA with a DNA helper plasmid, coding for both recombinases. DNA helper is more stable than mRNA and, therefore, lower concentrations are generally used in the injection mixes.

Although RMCE has been achieved in *Drosophila* (Horn & Handler, 2005; Oberstein et al., 2005), it not was achieved in diamondback moth using the same *lox* and *FRT* heterospecific sites. However, as only one line was tested, it is impossible draw strong conclusions about the system's function in this moth. The efficiency of cassette exchange has been shown to be influenced by the DNA surrounding the recombination sites (Nakano et al., 2001; Mlynárová et al., 2002). Therefore, it is critical to test a range of independent insertions of the recipient construct.

Nevertheless, regarding all three RMCE attempts, one can not exclude the possibility that unfavourable kinetics has not been overcome with these designs and that excision

remains favoured over integration. As a result, integration events may have been immediately reversed by an excision event, and therefore stable integration could not be achieved. In this study, all recombination sites were in direct orientation. A potential improvement to this design would be to use recombination sites in inverted orientation. If integration with subsequent excision occurred, as the sites are inverted, excision would be replaced by an inversion (Chapter 4, Figure 4.1). This may increase the likelihood of achieving a stable integration.

Besides testing the improvements mentioned above, there are other strategies that could be tested in order to achieve RMCE in diamondback moth, mainly using the Cre/*lox* system. For instance, the use of recombination *lox* sites with mutated symmetric elements instead of mutated spacers is another strategy to alter the specificity and directionality of the recombination reaction. Recombination generates one of the sites with two mutated arms that are no longer recognised by the recombinase (Langer et al., 2002). Two *lox* heterospecific sites with mutated symmetric elements have been engineered – *lox71* and *lox66*- and have been successfully used in to target sites in the chromosomes of plants (Albert et al., 1995), mammalian cell cultures (Araki et al., 1997) and *Drosophila* (Oberstein et al., 2005). One drawback of this strategy is that mutations in the symmetric elements weaken the affinity of the recombinase for its binding sites (Langer et al., 2002). Therefore, the use of mutated spacers was preferred in this study.

Analogous systems to the Cre and Flp system, such as Dre/*rox*, have also been described that merit consideration. This Cre-like site-specific recombinase has been tested in *E. coli*, as well as by transient expression in Chinese hamster ovary (CHO) cells, with results comparable to the Cre/lox system (Anastassiadis et al., 2009). More recently, two new site-specific recombination systems based on the Cre/lox system

have been developed - the VCre/VloxP and the SCre/SloxP - and shown to work in mammalian cells (Suzuki & Nakayama, 2011). If these were shown to work in diamondback moth, it could serve as another tool to use in combination with the Cre/*lox* and Flp/*FRT* systems for genome engineering in diamondback moth.

RMCE using the ΦC31 system has been reported in *Drosophila* (Bateman et al., 2006). This consists of a docking site containing two *attP* sites in inverse orientation and a donor plasmid with two *attB* sites. As with the Cre and Flp systems, the DNA fragments in between inverted sites can be exchanged. Since ΦC31-mediated integration was achieved in this study and the ΦC31 catalyses unidirectional integration - an advantage over the bi-directional Cre and Flp recombinases - this strategy might be an alternative to RMCE using the Cre or Flp systems. One potential disadvantage of this strategy, compared to the other two systems, is that two identical inverted *attR* sequences are generated that could be vulnerable to homologous recombination. If this were to occur, the line characteristic could change and render it unsuitable for its designed propose. This could be overcome if another integrase, with similar characteristics to that of ΦC31, were shown to be active in the diamondback moth. If that was the case, an *att* site from different integrases could be used for the generation of two different inverted recombination products, less likely to recombine. Potential integrases are the Bxb1 integrase (Kim et al, 2003), the ΦRv1 integrase (Bibb et al., 2005), the ΦMR11 integrase (Rashel et al., 2008), and the ΦBT1 (Zhang et al., 2010), which all belong to the serine family and have similar mechanisms of action to the ΦC31 integrase. In addition, these enzymes could be an alternative to the ΦC31 integrase.

A genomic manipulation tool such as RMCE would be extremely useful in insect trangenesis as only desirable sequences within the cassette are integrated. In addition,

it has the potential to integrate sequences that do not on their own produce a phenotype, as a successful exchange can be detected by the loss of a marker. These potential benefits would reduce the amount of exogenous sequences required for integration (Bateman et al., 2006).

However, while it would be desirable to be able to perform cassette exchange in a single generation with a single injection, the same final product can be generated by sequential use of tools that I have successfully tested. With suitable arrangement of attP and loxP or FRT sites, ΦC31-based integration of a complete plasmid followed by Cre- or FLP-mediated recombination can give the net effect of cassette exchange.

6.8. Regulatory sequences for the development of a female-sterile pink bollworm strain

One potential method of inducing female sterility, tested in this study, involves the use of regulatory sequences (e.g. promoters) from maternally expressed genes that are expressed during oogenesis. Three *B. mori* putative promoters of maternally expressed genes – chorion peroxidase, egg-specific protein and vitelline membrane protein 30 - were shown to be active in pink bollworm by driving expression of a marker gene exclusively in the ovaries of the females. In addition, the egg-specific protein putative promoter was able to drive expression of tTAV in a tetracycline-repressible manner, when driver strains (egg-specific protein putative promoter-ttAV) were crossed to a reporter strain (*tetO*-AmCyan). Two out of the eight egg-specific protein putative promoter-tTAV driver lines tested were also able to drive the expression of the pre-apoptotic gene $reaper^{KR}$ when crossed to the lethal strain (*tetO*-$reaper^{KR}$). However, the expressed $reaper^{KR}$ did not have a significant impact on

female fertility in one of the lines (OX4521C) and it was unclear if it had or not a significant impact on female fertility in the other lines (OX4521B).

Results show that the putative promoters tested were unable to express tTAV at sufficient levels, except the egg-specific protein putative promoter in some lines. Therefore, they were shown to be unsuitable for this type of strategy. Egg-specific proteins are probably needed in higher quantity than any other protein or enzymes, indicating that - for a strategy such as the one tested here - it is essential to use promoters of genes that express at high levels.

Alternative lethal effector genes should be considered. Other lethal genes such as NIPP1 and calmodulinB34 have been tested in pink bollworm without success. Other *Drosophila* pro-apoptotic genes, such as *the head involution defective* (*hid*), successfully used in the medfly (Schetelig et al., 2009), have not yet to be tested in Lepidoptera. Nucleases might also provide an alternative to pro-apoptotic genes. They cleave the phosphodiester bonds between nucleic acids, destroying the genetic material of the cells. If a nuclease gene is expressed under the control of an ovary-specific promoter, it might destroy the genetic material of the cells where it is being expressed, rendering the female moths sterile.

Alternative mechanisms, such as RNA interference (RNAi), might prove useful in disrupting oogenesis. Recently, Xu and colleagues (2011) characterised a novel maternally expressed *B. mori* gene, the *Bombyx mori* egg protein 80 (BmEP80), a component of the vitelline membrane that is secreted by follicle cells during late vitellogenesis to early choriogenesis. RNAi against it resulted in collapse of eggs. If the BmEP80 were shown to be active in pink bollworm, an RNAi approach could be used to induce lethality in eggs. Furthermore, this strategy could be applied to the candidate genes tested here. The vitelline membrane protein 30, with a similar

expression profile and function to the BmEP80, is a particularly a good candidate. RNAi against the egg-specific protein could also prove efficient as the egg-specific protein is considered to be the most important yolk protein for embryo development. Reduction in egg-specific protein production might have a significant impact in embryo mortality.

All genes used in this study are synthesised by the follicle cells, which are of somatic origin. As an alternative, putative promoters of germline-expressed genes, such as those expressed by the nurse cells, could be used instead of the somatically expressed genes tested here. The nurse cells are directly linked to the oocyte, synthesise maternal RNA and facilitate its transport to the oocyte in early oocyte development. Any deleterious effect on these cells would most likely cause a significant effect on oocyte development. Suitable candidate genes were not apparent when the present study was undertaken, however they could be identified by any of a range of molecular methods. The availability of a genome sequence for pink bollworm would greatly facilitate such identification; this is being undertaken by the Beijing Genomics Institute.

This study focused on the potential development of a female-sterile system. Sterile females would be a valuable tool in control programmes such as SIT. A female-sterile strain would contribute to eliminate the need for sterilization by irradiation that, due to the high doses needed for the Lepidoptera, tend to cause a decrease in the insects' performance (Seth & Sharma, 2001). Preventing eggs' development to larvae would be particularly useful in many agricultural pests, like pink bollworm and diamondback moth, where the larvae cause the damage. In addition, as no progeny are generated, no transgenes will ingress and propagate in the wild population.

Other systems with similar advantages to the female-sterile system, such as embryo lethality, could be tested in pink bollworm. In an embryonic-lethality system, a promoter active in early embryo development is used to drive expression of a dominant lethal which is passed on to the progeny, killing it at this stage. Such a system has been successfully developed in *Drosophila* (Horn & Wimmer, 2003) and *C. capitata* (Schtelig et al., 2009). In both systems the embryo dies and larvae therefore will not hatch, as it would occur in a female-sterile system. However, the characterisation of embryo-specific promoters functional in pink bollworm would be needed for the development of such a system. As previously mentioned (section 5.1.), a male-sterile system, that has not yet been developed, would also be a useful tool in pink bollworm SIT programmes. As pink bollworm females can mate several times over their lifespan, normal sperm function and quality would have to be maintained for adequate sperm competition with that of other males so females do not reject the sperm.

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6.9. Prospects for the future

The development of a genetic transformation system for the diamondback moth permits the genetic manipulation of this species for a variety of basic and applied purposes, and it is a key step towards the creation of strains potentially useful for the use in SIT programmes. Knowledge gained about *piggyBac* activity, gene regulatory sequences and fluorescent marker proteins will aid the development of transgenic strains such of strains with a heritable marker (such as those generated here), the development of genetic sexing strains, and RIDL strains.

Site-specific integration will allow for the direct comparison of transgenes in the same genomic locus. It will be a useful tool in gene function studies and could be used to understand mechanisms of insecticide resistance (for which this agricultural pest is notorious), and overall understanding of this species. The Cre/*lox* and the Flp/*FRT* system will allow the control of gene expression and production of strains without undesired sequences.

It is hoped that this site-specific recombination technology can be transferred to other lepidopteran species of agricultural importance, including the pink bollworm.

Although development of a female-sterile, repressible lethal transgenic strain of pink bollworm is incomplete, considerable progress towards proof-of-principle was made. While further work is clearly required, the potential advantages of a system that prevents egg production or development of larvae is clear, and warrants further investigation and investment. It seems likely from my work that suitable strains could in due course be constructed and these would likely be a valuable tool in advancement of SIT-type pest control programmes.

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Appendices

Appendix 1.

Sexing diamond back moth and pink bollworm pupae

1. <u>Sexing diamondback moth pupae</u>:

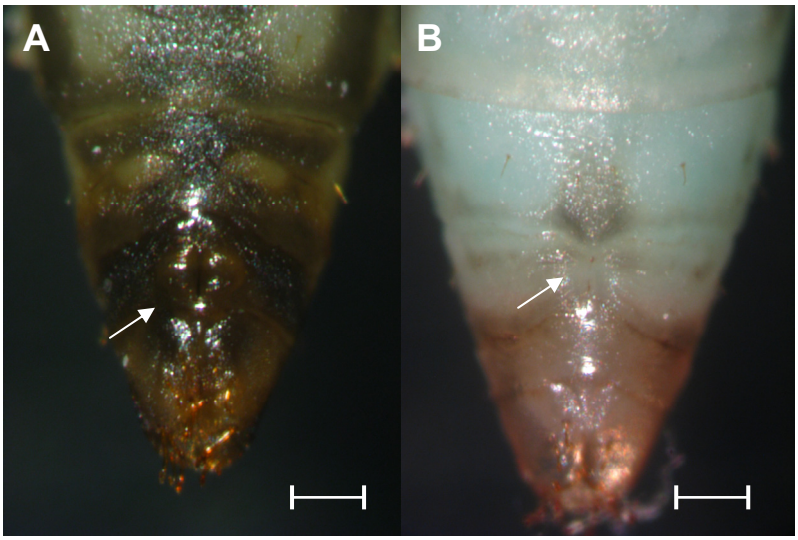


Figure 2. Morphological differences between males and females pupae used for sexing diamondback moth: males (A) are identified by the presence of two adjacent bulbous features on the ventral side of the last pupal segment (indicated by an arrow), whereas females (B) are identified by a dark groove on the same segment (indicated by an arrow). Scale bar represents 0.6 mm. (Pictures taken by Adam Walker, Oxitec Ltd.).

2. <u>Sexing pink bollworm pupae</u>:

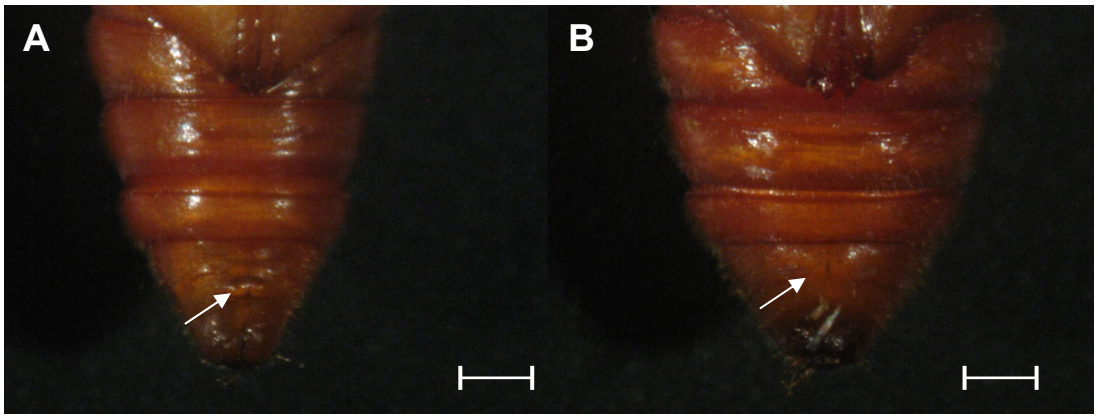


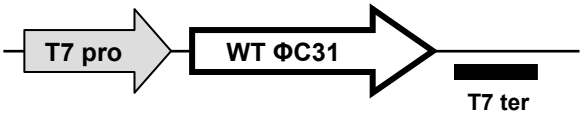
Figure 1. Morphological differences between males and females pupae used for sexing pink bollworm: males (A) are identified by the presence of two adjacent bulbous features on the ventral side of the last pupal segment (indicated by an arrow), whereas females (B) are identified by a dark groove on the same segment (indicated by an arrow). Scale bar represents 0.1 mm.

Appendix 2.

Maps of contructs used in this thesis and not descrined in the main text.

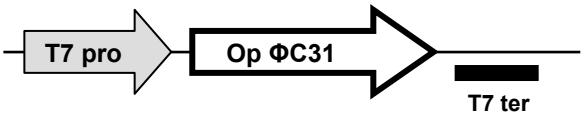
1. <u>OX3869</u>

The OX3869 helper plasmid encodes the wild-type (WT) ΦC31-integrase under the control of the T7 promoter (T7 pro). It also contains a T7 transcription terminator sequence (T7 ter).



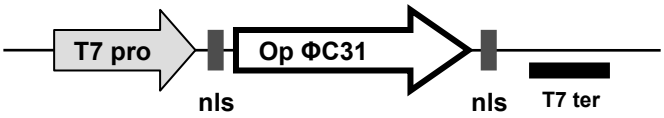
2. <u>OX4505</u>

The OX4505 helper plasmid encodes the codon-optimised (Op) ΦC31-integrase under the control of the T7 promoter. The integrase has been optimised for three species: *Bombyx mori*, *Drosophila melanogaster* and *Aedes aegypti*. The construct also contains a T7 transcription terminator sequence (T7 ter).



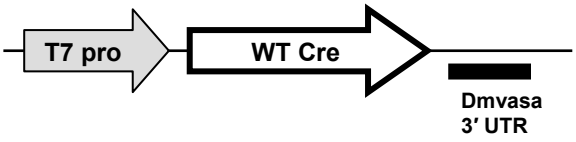
3. <u>OX4533</u>

The OX4505 helper plasmid encodes the codon-optimised (Op) ΦC31-integrase under the control of the T7 promoter. The integrase has been optimised for three species: *Bombyx mori*, *Drosophila melanogaster* and *Aedes aegypti*, and is flanked by two nuclear localisation signals (nls). The construct also contains a T7 transcription terminator sequence (T7 ter).



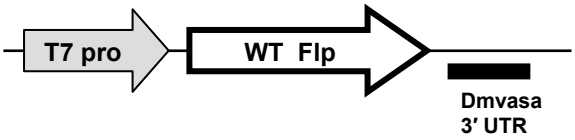
4. <u>OX4611</u>

The OX3869 helper plasmid encodes the wild-type Cre recombinase under the control of the T7 promoter. The construct also contains the 3′ untranslated region of the Drosophila melanogaster *vasa* gene (Dmvasa 3′ UTR).



5. <u>OX3608</u>

The OX3608 helper plasmid encodes the wild-type Flp recombinase under the control of the T7 promoter. The construct also contains the 3′ untranslated region of the Drosophila melanogaster *vasa* gene (Dmvasa 3′ UTR).



Appendix 3.

Primers used in this thesis.

Primer name	Sequence (5' →3')
pJETFP2	ATCAACTGCTTTAACACTTGTGC
pJETRP2	AAAGAAGAACATCGATTTTCCATG
1338	GTGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGTGT
1339	CGACACCGCCCTCCGCACC
1340	GATCACACCGCCCTCCGCACC
PB1	GGCGACTGAGATGTCCTAAATGCAC
PB2	CAGTGACACTTACCGCATTGACAAG
PB3	CAGACCGATAAAACACATGCGTCA
PB4	GTGCCAAAGTTGTTTCTGACTGACTA
PRIMER	GTGTAGCGTGAAGACGACAGAA
MID	GACGACAGAAAGGGCGTGGTG
K10R	CTGAACACCCACAGTACTGTGTACTACCACTG
SMA	TGTGCCAAAGTTGTTTCTGACTGAC
SV40F	CACTGCATTCTAGTTGTGGTTTGTCCAAACTC
SMB	ACTGAGATGTCCTAAATGCACAGCGAC
1000	CGCGCCATGCTAGCATATTTAAATATTT
1001	CGAAATATTTAAATATGCTAGCATGG
Int1	ACACGCTAGACCAAATGTGTTCTGTG
Int2	TCTCCCTTGCTACTGACATTATGGCTG
Int3	CTGCAAGGCGATTAAGTTGGGTAAC
Int4	CAGTTCGGTTATGAGCCGTGTGC
Int5	GACCGTCGAGAACCCGCTGA
ExcF	CATGATGTGTGACAGTGGTACGAAGT
ExcR	CGCTGCCAGTTCGACACCGT
Opie2R1	CATACTCGGTGGCCTCCCCAC
1301	CTACGTGACTGCCGACACGTAC
980	CTATTATCGATTTGCAGTTCGG
IE1R	CCCTAATGGCGAACACGATAACAATA
AmpRF	TCTCAGCGATCTGTCTATTTCGTTCATC
AmpRR	ATGCCTGTAGCAATGGCAACAACG
4319Nf2	TTCAGAACATCCACGAAACCACG
4319Nr1	GTCTTCCGTAGAGATAGAGAAACTTC
L1-F	GGCCGCATGGCGCGCCATAACTTCGTATAATGTATGCTATACGAAGTTATATA
L1-R	GATCATATATTGAAGCATATCGTATGTAATATGATTCAATACCGCGCGGTACG
L2-F	TAAATCCCGGGGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCATC
L2-R	GATCCTACTTCAAGGATATGAAAGATCTCTTATCCTTGAAGCCTTATCCTTGAAGGGGCCCTAAATTA
L3-F	GGCCGCATGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC
L3-R	GCGCGGTACTTCAAGGATATGAAAGATCTCTTATCCTTGAAGCCTTATCCTTGAAGTACG
L4-F	ATGGCGCGATATAACTTCGTATAATGTATGCTATACGAAGTTATATA
L4-R	GATCATATATTGAAGCATATCGTATGTAATATGATTCAATAAT
L5-F	TAAATGAAGTTCCTATTCCGAAGTTCCTATTCTTCAAATAGTATAGGAACTTC
L5-R	GGGCCTACTTCAAGGATATGATAAACTTCTTATCCTTGAAGCCTTATCCTTGAAGTAAATTA
L6-F	ATCCGGGATATAACTTCGTATAAAGTATCCTATACGAAGTTATATC

Primer name	Sequence (5' →3')
L6-R	GATCCTATATTGAAGCATATCCTATGAAATATGATTCAATATA
L7-F	CTAGGTAGCATGCATAACTTCGTATAATGTATGCTATACGAAGTTATAT
L7-R	CAT CGTACGTATTGAATCATATTACATACGATATGCTTCAATA TA TGATC
L8-F	TATGTAGATATCGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTA TAGGAACTTCTAGG
L8-R	CGCGCCTAGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG GAACTTCGATATCATCA
L9-F	CTAGGATGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGG AACTTC
L9-R	ACCAGGTTAGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATA GGAACTTCATC
L10-F	TAACCTGGTATATAACTTCGTATAATGTATGCTATACGAAGTTATATGCA TG
L10-R	CATATAACTTCGTATAGCATACATTATACTAAGTTATAT
L11-F	TATGATGAAGTTCCTATTCCGAAGTTCCTATTCTTCAAATAGTATAGGAA CTTC
L11-R	ACCAGGTTAGAAGTTCCTATACTATTTGAAGAATAGGAACTTCGGAATA GGAACTTCATCA
L12-F	TAACCTGGTATATAACTTCGTATAAAGTATCCTATACGAAGTTATATGG
L12-R	CGCGCCATATAACTTCGTATAGGATACTTTATACTAAGTTATAT
BmVMP30GSP	GGTCAGCTCGTTGCCACTTGTTTGCCTG
BmVMP30N	GTAGGTCGGAAACTGAGCCATGACCGGG
BmespRace	CCGCACGAGACGCCGAATCCGAATC
BmespRacen	GCAGGAGATGCTCTGCACCAGCGTCAGA
928	GGTGTGGGATCCCTCTTGTACGTGAATGTGTTCTTCATAAGG
944	(GGTGTGTCTAGATACTGACTACCGCTCAACAATAG
945	GGTGTGTCTAGA TACGACTTCAAGGAAATGTTATCTGC3
927	GGTGTG CGGCCG AT CCTAGG CGAATGATCGTTACGGGCAAGG
1004	GGTGTGCCTAGGGTCAGCAATGCCAGGAGCAGATC
1005	GGTGTGCTCGAGAATGGATCCTGCCAACAGTTGATTGTGTGGTAAAGG
BmVMP30AvrF	GGTGTGCCTAGGTCATTCAAATATCAATCGCAGGAGCTA
BMVMP30BamR	GGTGTGGGATCCTTTTCATTATACCGTAATTATGATTATGAATAAAG
BMVMP30xhoR	GGTGTGCTCGAGTTTTCATTATACCGTAATTATGATTATGAATAAAG
1075	GCGACGACGATGTTCGTGCG
1076	CGGTGGTGGTGAACGAGTAACCC
825	GTGCCAGCCCGCAGGTCTG
1315	CCGTCGCTTAATCGCGGCTGAG
ESPF	TCCTGCGCATTCCACCCACG
ESPR	TACGGAAGAGGGGCGAGCGT
VMP-RTF	AACTGATAGATTGCGTGACAGGCGAT
VMP-RTR	AACAAGGGTTGTAGGACGCCGTTT
NTPF	CGCGGATCCAGCATCAAACTG
NTPR	GATCTCTTTCTAGATTCGCTATTGC
852	CGTCAGGCAATCGAGCTGTTC
853	CAGTCTGCGCGTGTGTCCCG
2628	GCAGACATCAGTATGATCGGTCAG
2628	GTTGAGCTCCTCATCCTCAGTGTAC
963	GTGGCCTTCTACATCCCGGATCAG
964	TTACTGGCTCGGACGGCGGTAAC

Appendix 4.

Full genomic sequences flanking the *piggyBac* insertions of OX4389, OX4570, OX3441 and OX1138 lines in diamondback moth.

The canonical TTAA insertion sit is underlined.

Line	5 flanking sequence – TTAA – 3 flanking sequence
OX4389A	TGATCTCACCGGTGAAGTTTCTGATTGTGAAGGCATCGTATGTATTCCCGGCTACAATACTGTAGGTGACCACGGA TGCTGTAAGAAAGGTTGAGTTAAGCGTTGTCAGAGAAAAGGGGAGTGTTTGTGGAGGTCAATCACAAATGTAATT GAAGAAAACTAGACGACCGAATGGCGTAGTGGTTAGTGACTCTGACTACTGAGCCGAAGGTCCCGGGTTCGATTC CCGGCTGGGGCAGATATTTGTTTAAACACAGATATTTGTTCTCGGGTCTTGGATGTGCCCGTAAAATGGCAATAGG CCCGCCCCCTATTACATTGGGACTAACAAAAAAACACTCTGGCGAAAAGTGGGTGCAGCAATGCACCTCTGCCTAC CCCGCAAGGGAGTACATTAGTACAAGGCGTGAGTGTGTGTTTTTTTAGACTTTAAAGTATCAATTTTAAATGCCAC GACTAATAATATTAGCTAATCTTTGTTATTATTAACAATGCTTACCAGTATCAATATCTAAGGCTGTAACTTGAGTCA CCAACACATTGATATTAGCATTCTCAGCAATGGATTCTGCTACATAAGTGTCTTGAGTGAAGTGGGGTTGGTTATC GTTCTTATCAGCAATTTCTATTCTGAACACTTGTTGTCCAGAATTGTGCTTTCCAGGAATAAGAGCCGACTCGGAAT TATCTGTAGCTATTATCTTAATATTGTAGAAACTTCGCTCTTCTCTATCGAACATTTTGAGCGTTGTGATGTTTCCAG TCAAGGTGTCGATTGCAAATGGGTCTGACGCATCACCTAGCTCATATGTAACTTGGTTGTTAGCTGATGTACCGTCT GCGTCGAAAGCTCTCACTTGCATGACTTGAGTGCCAGGAGGTTCGTTTTCTAGGACCTGACCCGATCA
OX4389B	TCGATATCGGATGCCTCCCGCTCGGCAGATATCCTTCCGTGCGCCGGACGATATTCATCGACTACTACTTATTTATC ACTCGTCTGACTTTTAAACTGATACTTCGGCAGCTCGCTCCTTTACATAATAACTGTAATGCAAAGCGGCGGCCGG GTGCCGTTGCGACTAACCTTCGAGTCGTGTTTAAGCAACAAATCTGTTCTGCTAGATGCATTATGCATTCATTACTG TTGAATAATGTATCGGGAGGTGTCTTTTGTCTTGTTTACAATGAAACAGAAGCAGATATTTTGGTATTCATTCATTG GCATAAAATATTGTGGTTTTTCATTTGAAATTTTGATGTACCTAAGTTTGAGTGGCTTTACGAATTTGAATTTTGGG TCGTGGTTTATTGATGAAATTTTATAGGTACGTCATTTGAATACGATTCGAATGGATTGTGCTAGAAGTATACAAAT AGTTTCTATTACATCTGCACGTGGCAAGCACAGAGCCTTCAGTGTTTACAAGTCGCCTGGTTTGTCGACGCGTCGTC AAATTCCATCATAACATTGGAAGTAGGAAACTAAGCAATCTCTCGAGCAGTGTTTATGGAATCGAGAAATTGAATC GAATGCTCTCAGCTGTGCGGCAGTATTTGTAAACAGCAGTGGGGGGAGGGGAAACCGAAAATACTTCAAACAGA AATTGAAATGATGCCAACTGAGAGCAGACGGCTAGCTGCGCTCTCGTAAACTTTTCACGATACGACATCTCATTTT ATTCTCGTTTATCTGTCTTTATGCTCATTTAATTTATGGCCCACAAGCGCGCGGATGGCGCGCGGTCAACGTAATTG TGTCACAAATAAATCGTTTCCGTCGTCCGTCGACTTTTGTTGTACGGTTTCTGACGCTCCCATTTTGGATTAGGTTGT GTCGGCTAATTAAAACTCAGATTAACAGCCTTATTACGTGTTCGACTGTTCTCTAAAATGCATAATACATGAACAGA CATCGCATAAATAAATTTGGTATTTAAATCTATTTTTAAATTTATATGCAGCATTAACATTAACACACACGAA
OX4389C	TCGATATAGAGCCTAACCGTTACCATTTATTTTATGAGGTATCGAATCCTGTGATAGTCGTACTGAGTCCGCGTTGT ACTATGTCGAATTACGTCCAACGTACGTACGGACTAAAAAGTTAATGCAAGCCCTTCTTTTTAATTTTTGACAAGAT GTTAAAATGACAGCCAGTCAATAACAAAATTTTAAGGTTGCTTTACATTTATTTTTCATCTCCTTTACTGATAAGTGT AAAAAAAAACTAAACAATTGACAATCTAAGGAAGGACATGAAGGCATTTATTAAATTAATTGTAGTATTCTCTTCA AGTCAATCGCAAAAAGGTTAAAGGAAAATTCTTAAAGTCGTCGCACCCAAAACAAAATGTCTTGACATCCGTTGAA ACTTAATTGACAATACTTTAAATGCAGAACACGCGAATAAATTGTATTAAATTGTTCTTGGACATCGTTATTTGGCG ACTGATTCAAGTCCGAATATATTGCCATGGTGAAATGATTTTATCGGAACGAAGTTCACGGTATGTTATTATGTGTT TATTGAACTTACGCAACCTTTTTTTCGTCTATGACTGTGACTGTGTTAAAACAAAACTTCAGACAGCTTTTTTAAAA TGGAATACCATTGCGATTGCGACCAGCGCAAATAGAAAAAACTTGTGAGGAATTTATTTTTCTGAGTTTTCTAAGT GATGTCTGTCCTTCACATGTATCGGGGGTCTATTATAGTGCGGGTTGTTGGACCCCAACACAAGCAGTTGAAGGTC CAATCTAACACAATTAAAACCTTTTTGCAGCCAATCAAAAAGCACAATCCACCAAAGTATTTAACCAACTATAAACT CCATCACCATGTGTTTATGTTGCAAATTGAATGACGAACAGAAACTACGCTCTTGGTGGTGGATAAGGAAACGGA ACAGGAAACTTGGAGACAAAATGGCGAAAATATGGGCGAAGAAAAAGGCGCGAAAAACATGGGATGAAACACA AGAGCATAGAGAAAAGTTTAAGAGTCTATGGTCGAAGATGAAAGGGTCAAAGGAGGCCGGCGTCAATGCCAGTG AAACCAGTAATGTCGATAGTTATTCTTCTGATGATGATGAACCTAAGGGCTCCCACACATAAACACGACACGATGT CGCATCGTGCCACGACGCGGCGTCGTTTCACAATACGACGTCGCGTCGCGGAAATTTACGACTACCATCGTAAAAA AAACACGACATCGTAACGTAAAGTTCCGCGGCACGATGCCGTCGATACAACATTCATTCCCTAAAAATGGACCGCA GTAGACGCGTACTGCTTCTGCTGTTAATAAGAAGACGAAGAAAGAAAAGACGA

Line	5 flanking sequence – TTAA – 3 flanking sequence
OX4389F	TAAGTTTCTCTTAGCTCTCTTGTATCCGTGTCGCTCTTAAAAATTACCGTTTTCCTGTTTTAAGGGACAATGGCGCG CCCTAGTGGGCCGCGCCTTCCACCACGGTCCCCCGCACACCAGAAGAGCATTAGTTCATGTCCGCCTCCCGCCTT TTTCTTTCCTCGGGGTTGATGGGGTCGCCTCCCAACCTCGTGGTGCGTCCCAATACCAGGGGGGTCGTTTTTTGG AGGGGGCGGACCCCCAAGAAAGTGTTTCTCCCCCCCCTCTTTTCTAAAAAATATAATTAAAAACCTCTCCTTCTCT TCTGGAGCGTTTTCACCCCTCTAAAAAAAAAGCCGAAAAATGTTCCGAGGGACCATTAGAATTTTTCGCTGGGTT TTCTAGGATAGGTTCCCCAGAAAAACACCCCCCCCCACAAACACGTGTTCTGGAGAGGGTTACCAAGGTTTTAAA AAAGGTTTTAAATCACGCCCGGAAATCTTGGGGGATTAAAAGGAGAACCTTGGATTTTCCCATGCAGGTGGGAA TTTTGTAGGGAATTTTTAGAAAAGATAGGGCGTGTGGTTTAGCTGAAGACGCCAGAAAAGGCGTTGTCCGGAG GCCGGTTGTATTTCAGTGACCGTGGAAGTTTATGGACTGATGTCGATGCCCATGGGAGTAATAGCTGTGCAACA CAGCAAGTCTTTATCAGTTAGGCTGAAAATACAGAACATTCAGCTATCTTGCTTGCAAAATAATCAATAAAATAAT AATGTCGAATATCTTCATGTAGGCTACCAATATGGCTGATAACGTGATATCAATGAGTGTCATTCTGGTGATAAG TCAACGTGTGCTCATCAATTCTGGGTAAAGCTGTGTTCATAGATCTCTTTTGGCAAGTACAAGCGAGGAACTATC AAATGTGGCTCTTGAATTAAAATAGCTCTTAATGCGACGGTGTAAAGACAAAACTACTCGCTTGTTGAAGCCAGC TCTAACTTTATTTGGGTCCTACGACAATATTGACATGAAATGTTTACTCCGTAAGGTCACTCTATCAACAACTCTTG TTTGGATAGTTGATATTAGAGGATGGGGAGTAGAACAGCGTGTTAGAATGAGAAACTTTGATACCAAATGATAT TGCCATGCTGGTCATCCCAAATTCAAAATTAAATGATTTATTTCACAAGAGTGTAAATAAAGAAACTCTTCAAACA TATTTCCCCTGCTTAGGTCGTTATTACAATTTACACATACTTACAAAAAGGCACAAAATGTTATTTTTTTAAACTCT GCCATTTCCTAGATTGAGAATTTTAAATAATGTAGTTACGATACACGTGTAACCATACACTCATTCAAGCCCTACG GCGATGCCATAAAATTCACCAGTAAAAAACCTAGAGCTAGAAAATACTACTTGCTATACCGGCATGTTATCTCAA CCTGTTATTGATGTTGAGTCATTATGCTAATAGTGGACGAGACCTACTTCGATAAACTGTTAAGATATGTAACAA CTTAGCAACAACAGGTAGTTCGGGATATCGCTAAATATTTTCTTT**TTAA**AAATCTTTTTTTGTAATATTTGTGAGCG AAGGTATTTTACCCTCTCTACATTTGCAGTAAAATGAGACTCGCAGTCTAACGTAAACGTAAACATAAGATAAGT CGTGGTACCTACAGCTACGGAAAAAATAATTTCCTACACAATGGAGGAGACAAAAGGCACGAACGTGGAATAC GTGTAGCACGTGACTGCGATTATTACGTACTTCTTTATCACATTAATAGGTTATTCACACAAATCCAACTGAAGAA AAAAAACGGATGATTAATTTGATATTTGTAGGAAATATACCTCTAGCCTAACAACGGATGTCTCAAGACTTTTTTC GCCCTACCGTCTACCTGTACGTAGGTACACTTTTCTCATAATAATATACTTTTTAGTGAATTTCAAAATCAACATTA CTAGTAAAACACCCTTTAGTCTTACTTGATTAAATAGTTAAGCTCTCCGCAGGTTTTCTAGTCACGAATTCGAAAT TTCTAATCTCTTGGAAATGTTCAAAGTCACACCAGCTATAACTACTGTGCCAACCACCCCAATAGCAGACGCAGG CTATTATAAACCTAGAGACTTTGCAATTAGGGTAAACTCACCTTCTGCATTGCTCTGGAGGGCACTTTCTCCTATA AGTTTAACGGTTAAAGGATGGTAATATTGAAACTCGATCA
OX4570A	TGATCACTATTTATTTTGTTAGAACTACGGAATGGACTTCGATGAAATTGGGCAATTCTATCCATCTTTCGTAGAA CAACGCGGACTCGGGTGTACTCTACTCATTGTATACCATGTGTCATTCCCACCCTATTGGCCCGACGACAACACTG CTTTGCGAAGTCGCATCTTATAAGTCCACGACTGTTTCGGACAGTTTTCAGGACAAATGCTTGGCTGTCGCACGTT TTTGCGATGTTGCAACGCAGTGTTGTGTTCCGGTTGGAGCGTAGCGAACTGTATAGTGAGGAATATATGCCCTG GAGGTTTGCTCGTAGCTCTTGGAAGTAATTATTTCGTTTGA**TTAA**ACGTAAAATATGGTGTGATCCATGTATTTTC TCAATCACAGCAGAAACGAAAGAGGACGTTTTCCATAGACAAAGATATAAAACTTAGACAAATCATCGA
OX4570B	CTCATAGCATTTGATGCGATTGGCAGTTTGCATATAAATAACCCTTGCATAGGTAATTCTTGCAATTACTCCGCCC GTTATTTGAAATACCTACATACCTACGTACATTTTACTTCACAATAGCACACATCTGCATAGTATACGTATCACGTC TATAATCCCTAGGTACAGTAGGTACTAGGTACACCACACGTCAAGCAACAGCAACTGGC**TTAA**
OX4570C	TCGGTGAAAAGAAATCGCGAAAACTATAGTGGATTAGTTCTTTCTTCCTCGGTGCTACAAAAGGTATTTTGATAG TTGATATCACTACGCACAGACCCA**TTAA**GGAAGTAATATATAAGTTGGAGGGGAAATAAATTCAATTTAGCTAAT AAAATCACCTGGCTTCACCAGTAGGTCAATAAGGGTGGACAAATAGAATTCCTCGGCCCTACCTCACAGATTAAA TTGGGGAAAAAAATTGTTAGGTGTAGGAAGTGTAGTCGATATACCTTCACTTCTTAAATATTGATCTAGTGATAAT AGTAAATAACATGAAGTGTTTTTTTGATCA
OX3441A	GACGACAGAAAGGGCGTGGTGCGGAGGGCGGtgTGATCAGCTGTTGCATTgTAAGGATCTAGTTAACTGTCACA AGGACAGCAGATTTTAGTATTGTGTTCAAATGAGATGACCTTTTGAGGATATTTATACGGTAAGTGATACCTAAG TTTTTTGTGCTTTTTTAGTTGTTGATAGTCTAAAGTTGTATTGTTTAAAGGATGCTGTTATATGAGAAGAATTTTAG GACCTTGTATGGAATGATGCATTGTATGTTGTATTCGT**TTAA**
OX3441B	TCGATGAACAATAAACAAAATACGGGTATAATAAATTCATATAGAAACTTACGAAAAACGTGTCCTGAAATACTA TTTGATTTTTTTGCAGCTGCTTTTACATACTAGTGTTTTTCTATAGTTTAGAAAGTTAAGTTATCACTTTA**TTAA**

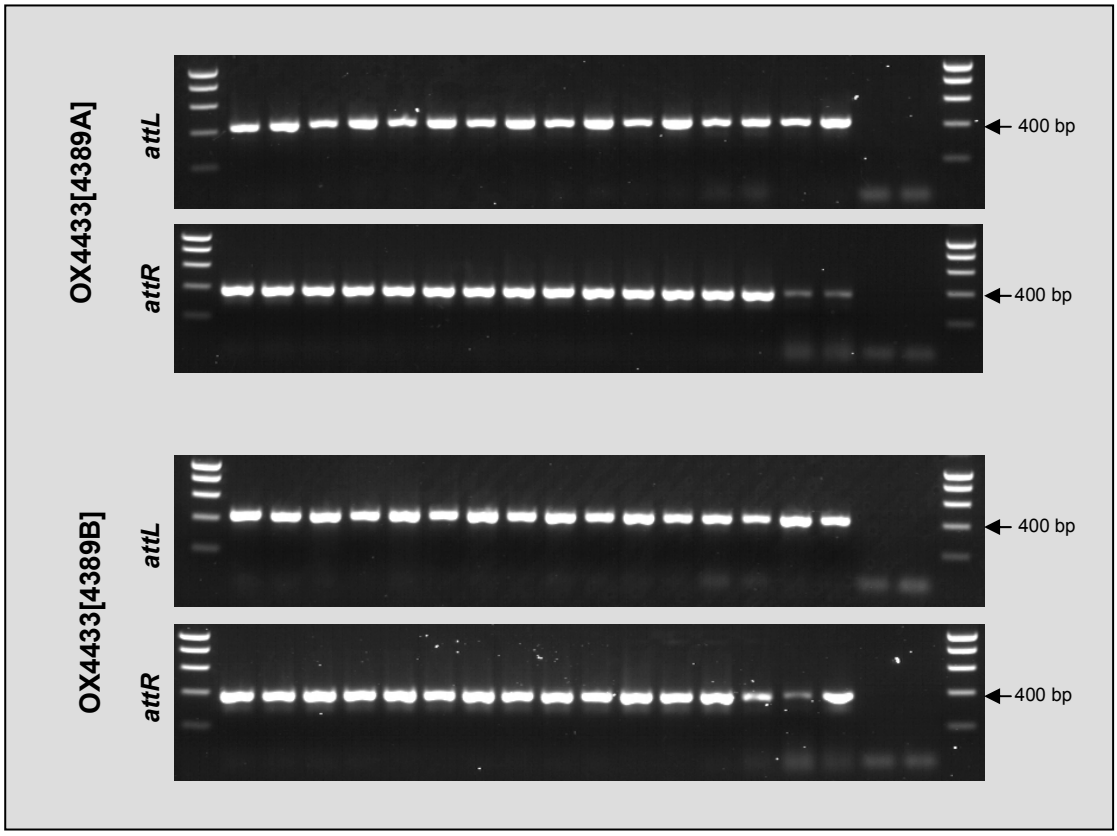
Line	5 flanking sequence – TTAA – 3 flanking sequence
OX3441C	GCGTAACGATCTTAGCTCTCATTCGTGAGTTCGTGGGTTCAAATCCTATGCCATGTACCAATGAAATAGAAATAA AAAAAAATTAAGTGTTTTTATAACACGTACTATTACGACGAAAGAAAATATCGTGAGGAAACCAGCACACCTGA GTATGTCAACCTACCAACCTGCAGTGGACCAGCGTGGTGGGAAATGGTTCAAGCTCAGGAAGGCAGTTGAGAC CTTGGGGATATGTACAAAGGTTTCATTCGAGAGAGCCAGATGCAGGTTCTAATATCCCCATAGAGAATGGAATA GTTTTTATTATTGTTCCGAAGAGAATCCCGCTTTCTTACTCAATACAGAGCAAGAGCTAGATAGAAGCAAGTTCA GGAACTTCATTCGTTCTACAGAATCTCCCTTTCCCAATGGTATAAATTGTAC**TTAA**AATGAATGTAAGCACTTTAT CAACGAAATCTTTGGGAATATTTCGCTCATCAGCATTTTATTTGAGCAGGAGTCCGAGATGCCCGGGCGA
OX3441D	**TTAA**GTGAGTAGATAATTGTGTCTTAATCGCAGACACTTCAAAGTTTTTTTTAAACTCAAGGTACCTGAGTATTCA ATATTTATGTACTAACTTAAGTATATTATTATTATGATGTAAAAAAATAAGGCTAACTATTTCTCTTCATTACACCC AAAATGGCGTTTTTTTTCAGGAAGATTTTTTCACTGTGGATCCAATTTATATAAAAATATACCTACTATTCTGAAAA AAAAAATACTTTTTCCAATGAAACACCAAAACCAACTAAGGACCTATCTTGTGACCAAAATCCCTTGTCTACATAG CCATTCTTCAGGTATCAAAAGAGACCATTATAATTTGACCCCTGGCATTCAAAGAGATCA
OX3441G	TCGGAAAACTCTGCATTACTTGGCACCCAGGCCACGAAAAAAGTCTGTGTGTCAGACACCCATCGGGAATCGAT ACCTAGAGGTATAATACGGACAGGGTACAGTATATATTATTCTACAACTTCTTATAGTATGTAATGTACATAACTA ACTGTAGGAGCCTTATAAGAGCAAAAGCTATTACCTATTAGCTGTAAGCTACAAATTCCGCATGGCTGATCAGTG TGACCTTGTAAGCTTAAATCGAAACGTCCATATTCATGAAATTATTGCCAATATCACATCGGTGTTGCCTGGAAAT GAATATCGGTGGTTCTCTGAAGTACGTAT**TTAA**
OX3441H	TGATCTAGACATTCCACTGAAGATGCAGTGTTAAAGTTAACTTCTGAAATCACAAAGCACCTAGATGATGGGAAG AAATGCGTCGGTATATTTCTTGATCTTCAAAAAGCATTTGACACTGTATCAATACCTATTCTGCTCAAACGCTTAG AAATCCTAGGTATAAGGGGCTCAGTGCTTGACTGGTTTGAGAGTTATCTCAGTCATCGTAGACAGTTCGTTCAAT TGGGAGGGTTTTCCAGTGATGAAGCGATCTGTACCTACGTCACAGCGTGGTTGGAAGACAGTCTCCTTTCGTTAA ATACTGCTAAGACTAAGTACCTATGCTTTAGCAAAACGAATGCATCTATGCCAACCAACGACTTCAGCATAACAA TACACTCGTACCCCTGTAACGCTTCTAACTCTCATAATCCCGATGAGTGCCACTGTTCTGTGCTTGACCGTGTCAA TGTAGTCAGATATCTAGGCGTATTGATAGATGATAGACTTCAATGGGTCCCTCACATAAAATCAGTGGCTAAAAG ATTACGTAAACTCATTTTCGTCT**TTAA**AATGAATGTAAGCACTTTATTAACGAAATCTTTGGGAATATTTCGCTCAT CAGCATTTTATTTGAGCAGGAGTCCGAGATGCCCGGGCGA
OX1138A	GAAATAACCCAACATGTTTTTATGGTAGTTATAGGATCAATAGCTTTTACAGCTGACAAGGCGCCCAAGAATGTT AACACGTTTATCTAATCAACGTCTTAAAAGCTATAAATAATGGCGCTTAATCCAAAAAAAAGGCTTCATATCAGG TTTTGATTTATTACTATTTCCAAAGATAATACAGGGTGTCGAACAAAAAATGAAACTAAGTATCATAGTCTCATCA AAATTACCTTCAGTGCAAAAAAAGCGATAGTGGATGTACAACGGATCGTATTGCATATGGATGGATCATGATTC GTACGATTATTCACTTGAGGCTATACAGTATATTAAAATACATATAAAGGCAGTACATAATGTTGTCAACTTGGTT TCCTGGTCTCGAGACGACCTTGCACCTGACGACTCTGATCTCGGTCAAGCGACAGTGACGTCATCCACTTCGACC CAAGATGCGCTCACGGACCAAGCAACTAACTATTTAAACACAAGCCTTTTAAAATCAAAAATAAAATGGAATGCC AACTGGACGACCTTCTCCTCTGGCGAACAGGATATGAAATGTATAATATATTCAAGAGAAAAAACTGCACGATTA TCGCCAAAAAACAACAAAATAAAGCAGATACCAACACAGATGTTCTAATGTGCCCAAAGAATGTAGTCCATTGG AATTTCCGTGGTCATTATCATGATTTCTACATTTCTACACCAGTTATGTAACTCGTGATATTCGTGGGTTTATTGAA GGATTAAGTTATTAAGATGCTACAAAATGTCAGCGAAAATCCTTATGAAGCCTTTTTCTTAAATATTTTATTCCTG GTGCGACGAACTTTTTTATCGATACCAACAAAATTACAAAACTTCTTGT**TTAA**CAAAGTGTTTACTTAAGTGGCTT CGTCACATTTGGAAGTGTAAACAATTTGTTTGACATCTGTCGAGATTATAGTCCTTTAAACCCGGATATTTATGGC AAAATACAGTGACTTTTAAACGGTTACCAGTTGGGACAGATTATTATCCATGGGCAAAAAATCTGTAGAGCAAA GCATAATAAAAATATTTTATGCATTGCTGAAGTAAGGTGAGTTAAGTATAATTAATATGTAAGGGGGTTTTAAGT TAATTTTTATGTTCCCCAACGTAGGTTAAGAGTTTATGTTTACACTAAAAATCAGCGTAAACTTCAAAAGTAATTT GAGTGAGTTCGTTTATAGTAGAACGAATTCCCTCATCTCCTATGTCCCACGGGCTCAGCACATAAACGCGGTCAA CAAAGACTGGTTCACAGTTCCCAAACGAATTCCAAGTGCTCTCCGAACTATCAAGCCGGCTTATTATTCATAGAG GAAACCGAGTAATTAGGCGTCACTCGTAAAACAGTCATCAAACATGTGTCGGAGTGTGTCGACGTAAAACCCGA CCGCTGCATAAATAATCCTGAGTGTGACAGGGCGGGCGCTCGCTGCTGCCTTTACGATCACACCGCCCTCCGC
OX1138B	GTGATCCTAATCGAGAAGGTAGGTACTTTTTGCCCCTGTCTCTACGCTGGCCTCATTACCCGTGGGACTGTAGGT ATACATATAAATCATACAATACATAATTAGTCATATTATATTATGTATGCCGATGTCTACAGGAAACAAAATTTCG ACAAGAATTTAATCCGAGAAATTTAATATCATATTCCTAGAAGTTACTGCTATAGGCACGTATATCGTTTTATTTTC C**TTA**

Line	5 flanking sequence – TTAA – 3 flanking sequence
OX1138C	GAACAAAATATCAGACATTTCAGAACCTGTATCTTTGCACCAATGAAACCTAGAGAGTTGAATTAAAAGTAGAACA TAAGTAAATTTTGTCTTCTTTATAAAAGCTATAGAGACCTTTCTCGTTAGAACCTTTCTACTAGCCCTATTTTGCATA AACAAAAAAAAAACAAGTTATAAAACTGATTTTTAGATAGAAATTTAGGAAATATTGTCTAATTGAAGGCAGCCAT AAATAATTTTCATGTTAGAGCCTTCTGAATATGGCTATTTAATTCATTTACATAAAACAAAAAAACAAACATAAAAA TATGCCCGTATTTCAGCTATTCACTATAAGTATATAGTGCCGTCGTGTAAAAGTGCTGCAAAATTGGATTTATAATA TGTGAAGGATCCCAAACATATAAAAAATGAAATTATTACGCAATTATTGCAATTTCAATATACTTAACTTGTCTTAA ATGACACAATAATGGCCCTTACTAGGTTATGAAATGGTCAGTGAGGGCGGTTTTAGGGTGAGAAAACCATATAAA ACCTAGTAAGTGTTTTCTTTTGTGCATTACAGCAACAATTTTAGTCATATTTAAATGAAATAGTATTGTATATAAAGC CTAGGTTCTGCCGCTTCTTTTAAGCTTACATTTGTTCAGATATCTATCATAGTTTTACCAGGGCATTGAAATGAAGGT ACAAAAACGTTTTTTGGCTCTCCTGAACATTTTGTCTCATGGCTGTTACTGTGTTTTAAAATGGGACAGCCGCGGTG **TTAA**
OX1138D	GTAAAGGCAGCAGCGAGCGCCCGCCCTGTCACACTCAGGATTATTTATGCAGCGGTCGGGTTTTACGTCGACACA CTCCGACACATGTTTGATGACTGTTTTACGAGTGACGCCTAATTACTCGGTTTCCTCTATGAATAATAAGCCGGCTT GATAGTTCGGAGAGCACTTGGAATTCGTTTGGGAACTGTGAACCAGTCTTTGTTGACCGCGTTTATGTGCTGAGCC CGTGGGACATAGGAGATGAGGGAATTCGTTCTACTATAAACGAACTCACTCAAATTACTTTTGAAGTTTACGCTGA TTTTTAGTGTAAACATAAACTCTTAACCTACGTTGGGGAACATAAAAATTAACTTAAAACCCCCTTACATATTAATTA TACTTAACTCACCTTACTTCAGCAATGCATAAAATATTTTTATTATGCTTTGCTCTACAGATTTTTTGCCCATGGATAA TAATCTGTCCCAACTGGTAACCGTTTAAAAGTCACTGTATTTTGCCATAAATATCCGGGTTTAAAGGACTATAATCT CGACAGATGTCAAACAAATTGTTTACACTTCCAAATGTGACGAAGCCACTTAAGTAAACACTTTG**TTAA**

Appendix 5.

Representative PCR analysis of 16 individuals derived by ΦC31-mediated integration of OX4433 into OX4389A and B lines.

The last two lanes on the left are negative controls. Smart ladder is run at both ends.



Appendix 6.

Mendelian inheritance of the transgene in OX4540 lines.

Chi square (χ^2) analysis to determine if transgenic to wild-type ratios significantly differ or not from 50:50 (df=1, p>0.05).

Line	Transgenic	Wild-type	χ^2	p value
OX4540A	136	112	2.323	0.1275
OX4540B	184	160	1.674	0.1957
OX4540C	200	140	10.588	0.0011*
OX4540D	164	176	0.424	0.5152
OX4540E	112	132	1.639	0.2004
OX4540F	136	120	1.000	0.3173
OX4540G (+)	-	-	-	-
OX4540H	104	164	13.433	0.0002*
OX4540I	136	144	0.229	0.6326
OX4540J	80	92	0.837	0.3602
OX4540K	152	108	7.446	0.0064*
OX4540L	144	132	0.522	0.4701
OX4540M	140	124	0.970	0.3248
OX4540N	280	168	28.000	0.0001*
OX4540O	148	116	3.879	0.0489*
OX4540P	112	96	1.231	0.2673
OX4540Q	120	168	8.000	0.0047*
OX4540R (+)	-	-	-	-
OX4540S	168	144	1.846	0.1742
OX4540T	184	148	3.904	0.0482*
OX4540U	148	148	0.000	1.0000
OX4540V	82	96	0.348	0.2940
OX4540W	192	176	0.696	0.4042

(+) Founder individuals of lines OX4540G and R were sterile, therefore, produced no progeny.

* This difference is considered to be statistically significant

Appendix 7.

Cre-mediated excision raw data.

Cross	R+G	G	WT	Excsision (%)	Cross	R+G	G	WT	Excsision (%)
1	0	42	37	100.0	**43**	0	2	100	100.0
2	0	42	114	100.0	**44**	131	60	144	31.4
3	0	64	44	100.0	**45**	36	106	110	74.6
4	2	63	58	96.9	**46**	204	0	42	0.0
5	0	13	14	100.0	**47**	57	21	51	26.9
6	0	237	64	100.0	**48**	198	0	43	0.0
7	1	74	97	98.7	**49**	45	10	65	18.2
8	39	32	79	45.1	**50**	164	36	53	18.0
9	0	94	84	100.0	**51**	129	8	31	5.8
10	42	126	135	75.0	**52**	103	101	84	49.5
11	0	129	1	100.0	**53**	122	4	47	3.2
12	15	110	117	88.0	**54**	0	3	0	100.0
13	0	117	48	100.0	**55**	200	35	0	14.9
14	0	115	189	100.0	**56**	130	12	133	8.5
15	0	327	109	100.0	**57**	56	42	82	42.9
16	0	141	96	100.0	**58**	113	1	71	0.9
17	17	145	121	89.5	**59**	30	0	21	0.0
18	35	101	114	74.3	**60**	65	1	38	1.5
19	42	28	74	40.0	**61**	51	0	0	0.0
20	0	89	79	100.0	**62**	71	35	34	33.0
21	0	168	131	100.0	**63**	73	12	18	14.1
22	31	92	74	74.8	**64**	138	7	31	4.8
23	11	51	62	82.3	**65**	68	29	88	29.9
24	11	73	84	86.9	**66**	48	13	51	21.3
25	0	28	15	100.0	**67**	72	13	34	15.3
26	0	51	38	100.0	**68**	32	22	27	40.7
27	1	148	1	99.3	**69**	67	0	33	0.0
28	12	11	20	47.8	**70**	62	0	1	0.0
29	1	1	1	50.0	**71**	129	4	104	3.0
30	14	59	64	80.8	**72**	132	22	137	14.3
31	10	47	73	82.5	**73**	52	20	66	27.8
32	5	36	40	87.8	**74**	49	64	30	56.6
33	0	29	24	100.0	**75**	52	31	37	37.3
34	3	62	4	95.4	**76**	0	8	0	100.0
35	16	40	36	71.4	**77**	6	55	78	90.2
36	4	34	15	89.5	**78**	1	135	131	99.3
37	4	20	14	83.3	**79**	0	37	45	100.0
38	7	49	51	87.5	**80**	34	129	0	79.1
39	0	128	1	100.0	**81**	0	0	0	Sterile
40	0	0	0	Sterile	**82**	91	0	95	0.0
41	16	13	19	44.8	**83**	54	0	0	0.0
42	3	42	73	93.3	**84**	84	61	46	42.1

Appendix 8.

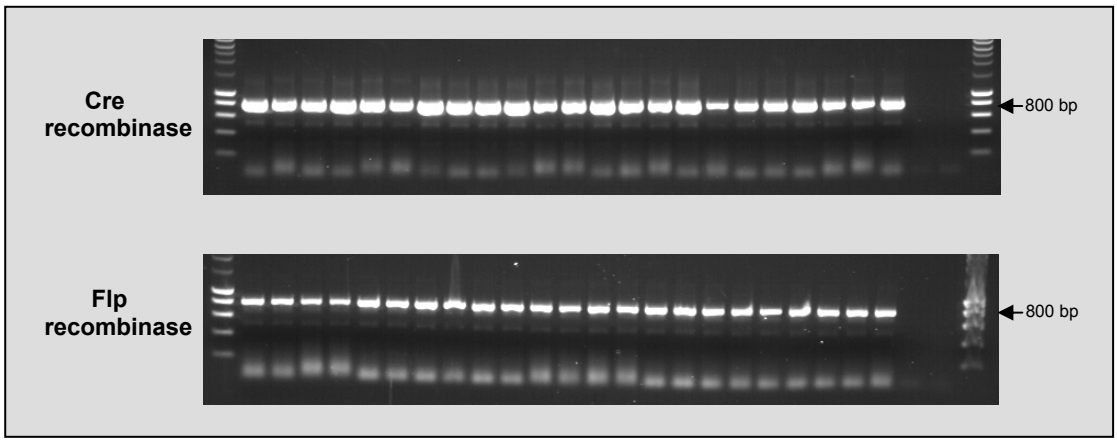
Flp-mediated excision raw data

Cross	R+G	G	WT	Excsision (%)	Cross	R+G	G	WT	Excsision (%)
1	0	110	101	100.0	**34**	61	113	186	64.9
2	0	150	136	100.0	**35**	0	214	163	100.0
3	0	99	78	100.0	**36**	135	185	129	57.8
4	0	125	103	100.0	**37**	0	196	141	100.0
5	0	303	0	100.0	**38**	1	237	148	99.6
6	0	65	56	100.0	**39**	42	131	136	75.7
7	0	74	67	100.0	**40**	7	200	106	96.6
8	0	123	102	100.0	**41**	2	131	119	98.5
9	0	18	17	100.0	**42**	131	54	121	29.2
10	0	133	0	100.0	**43**	218	105	115	32.5
11	0	73	73	100.0	**44**	321	48	17	13.0
12	0	70	71	100.0	**45**	75	163	184	68.5
13	0	57	64	100.0	**46**	192	8	105	4.0
14	2	70	73	97.2	**47**	163	23	168	12.4
15	0	13	12	100.0	**48**	290	102	58	26.0
16	0	99	100	100.0	**49**	212	1	71	0.5
17	0	246	19	100.0	**50**	321	20	65	5.9
18	0	145	0	100.0	**51**	280	61	106	17.9
19	0	161	179	100.0	**52**	184	63	132	25.5
20	0	132	0	100.0	**53**	142	82	63	36.6
21	0	146	156	100.0	**54**	S	S	S	Sterile
22	0	133	120	100.0	**55**	0	196	0	100.0
23	54	61	114	53.0	**56**	S	S	S	Sterile
24	9	78	80	89.7	**57**	0	14	16	100.0
25	0	88	86	100.0	**58**	0	17	24	100.0
26	0	107	95	100.0	**59**	6	307	6	98.1
27	1	73	72	98.6	**60**	0	117	29	100.0
28	3	72	90	96.0	**61**	43	161	178	78.9
29	0	92	60	100.0	**62**	244	80	43	24.7
30	0	213	0	100.0	**63**	78	0	56	0.0
31	3	80	72	96.4	**64**	0	118	93	100.0
32	70	74	139	51.4	**65**	162	2	52	1.2
33	1	268	125	99.6					

Appendix 9.

Representative PCR analysis of 24 individuals derived by Cre and Flp-mediated excision of the *Opie2*-DsRed2 marker from OX 4548[4540A] line.

The last two lanes on the left are negative controls. Smart ladder is run at both ends.



Appendix 10.

Test crosses data for graphs in Chapter 5.

4360A × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	290.6	4.39242	0.8447	0.01588
♀ R+G (T)	310	10.41415	0.8151	0.01345
♀ G (NT)	300.3	7.92296	0.7926	0.02733
♀ R (NT)	291.8	10.66854	0.8287	0.02107
WT	288.1	7.92528	0.8384	0.01562

4360B × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	288.6	9.02490	0.8070	0.02917
♀ R+G (T)	286.2	9.30328	0.8183	0,02967
♀ G (NT)	285	4.51418	0.8168	0.03230
♀ R (NT)	291.6	11.42337	0.8621	0.02263
WT	311	11.39318	0.8265	0.01662

4360C × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	246.3	3.79781	0.8098	0.01983
♀ R+G (T)	286.8	6,06529	0.8402	0.02224
♀ G (NT)	273.2	6.52142	0.8076	0.01928
♀ R (NT)	265.8	7.22003	0.7782	0.01575
WT	287.4	11.69444	0.7970	0.2650

4360D × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	375.2	6.11882	0.9447	0.01588
♀ R+G (T)	372.3	8.81167	0.9248	0.01925
♀ G (NT)	371.3	13.68621	0.9045	0.01790
♀ R (NT)	361.1	9.24896	0.8909	0.02654
WT	388.2	13.46419	0.9262	0.0900

4498A × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	210.4	5.74688	0.8098	0.02053
♀ R+G (T)	232.2	8.79247	0.8003	0.01716
♀ G (NT)	231.7	12.24931	0.8820	0.01243
♀ R (NT)	239.4	9.00295	0.8064	0.01876
WT	249.3	7.73455	0.7700	0.01352

4498B × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	215.4	6.73003	0.8792	0.00719
♀ R+G (T)	215.9	5.58287	0.8801	0.01203
♀ G (NT)	271.9	10.63689	0.8457	0.01293
♀ R (NT)	279.9	4.45583	0.8468	0.02141
WT	296.4	7.14330	0.8690	0.01067

4521A × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	230.4	5.9613	0.8532	0.01276
♀ R+G (T)	269.6	7.07295	0.8324	0.01137
♀ G (NT)	252.1	9.65338	0.8156	0.01540
♀ R (NT)	249.2	3.84939	0.8184	0.01540
WT	249.6	9.84683	0.8365	0.00063

4521B × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	144.2	2.24499	0.8333	0.02536
♀ R+G (T)	203.0	3.08761	0.8235	0.01618
♀ G (NT)	215.1	5.12608	0.8728	0.01542
♀ R (NT)	217.2	7.63006	0.8191	0.01575
WT	234.0	7.94285	0.8346	0.01344

4521C × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	294.7	11.48530	0.8794	0.01665
♀ R+G (T)	271.9	10.63689	0.8605	0.01279
♀ G (NT)	293.2	11.55162	0.8745	0.01494
♀ R (NT)	299.7	9.91525	0.8556	0.01744
WT	284.9	10.71598	0.8839	0.01612

4521D × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	291.9	5.40874	0.8560	0.01383
♀ R+G (T)	298.1	13.01149	0.8863	0.01153
♀ G (NT)	297.9	7.26552	0.8745	0.01544
♀ R (NT)	302.2	4,81156	0.9020	0.01386
WT	295.3	8.18542	0.8594	0.00663

4521F × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	259.2	14.22033	0.7466	0.05917
♀ R+G (T)	259.1	7.22257	0.8818	0.01203
♀ G (NT)	244.2	7.67362	0.8707	0.00987
♀ R (NT)	253.5	11.46613	0.8916	0.01289
WT	243.9	8.60678	0.8782	0.01099

4521G × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	265.3	4.11245	0.8733	0.01640
♀ R+G (T)	272.9	7.76237	0.8370	0.01322
♀ G (NT)	269.4	7.88134	0.8413	0.01775
♀ R (NT)	272.6	9.53263	0.8452	0.02312
WT	252.8	8.50464	0.7284	0.01095

4521J × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	150.9	5.87358	0.7457	0.01403
♀ R+G (T)	200.2	5.92181	0.7323	0.02185
♀ G (NT)	173.5	5.60208	0.7208	0.1981
♀ R (NT)	183.5	6.91481	0.7037	0.02170
WT	179.0	6.56083	0.7225	0.01699

4521K × 3069C				
	Mean eggs laid	**SE**	**Egg hacth rate**	**SE**
♀ R+G (NT)	182.6	7.12772	0.7770	0.03627
♀ R+G (T)	201.8	7.48599	0.7861	0.01690
♀ G (NT)	186.4	4.05024	0.7628	0.02149
♀ R (NT)	190.6	7.07138	0.7383	0.03968
WT	115.7	3.2991	0.7794	0.01437