Biotechnological approaches to fight fruit flies of agricultural importance

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by

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Declaration

I declare that this doctoral thesis titled “Biotechnological approaches to fight fruit flies of agricultural importance” was a product of my research work at the Department of Developmental Biology, Georg-August-University Göttingen, and that it has not been submitted elsewhere for the award of any degree. Works of other people cited herein have been acknowledged by reference.

Dedication

To HE who makes all things possible

Acknowledgement

My sincere gratitude goes to my family for all their support, understanding and encouragement that kept me going during the whole period of this doctoral study.

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ABSTRACT

Tephritid fruit flies destroy fruits and vegetables, causing tremendous economic losses and bringing a barrier to trade in fruits, vegetables and their associated products. The Sterile Insect Technique (SIT), a target-specific and environment-friendly pest control method, has been used to combat several insect pests for over five decades and has proven to be quite effective against tephritids. Basically, SIT requires mass-production and mass-release of sterile insects to reduce the wild population of the same species. Sex separation prior to release is important in SIT for many insects as male-only release has been shown to achieve a more effective population control. Post release, the wild population and their released sterile counterparts need to be effectively monitored to ascertain the success of the programme. While SIT has achieved great success, improvements to the various technical aspects are constantly made as that will shape the method into a highly efficient, safer and cheaper programme to execute. Biotechnology has a great potential to bring desired improvements to SIT in ways that may be difficult to achieve by other means.

This study aimed at an improvement of SIT using molecular biotechnological approaches. An early-acting sex separation system based on female-specific embryonic lethality was developed as a transgenic sex separation alternative for SIT in the Mediterranean fruit fly *Ceratitis capitata* using a sex-specifically spliced intron in a proapoptotic gene driven by a conditional heterologous transactivator under the control of a blastoderm-specific promoter. Transgene modification by site-specific recombination in the sexing effector line was further performed and subsequent stabilization of the transgene by transposase-induced partial removal of transposon inverted terminal repeat (ITR) achieved to make the line safer for application in operational SIT programmes. The strategy used for transgene stabilization also provides an avenue to combine other transgenic systems with the early-acting sexing system and to improve the efficiency of this site-specific recombination strategy in medfly, the upstream region of the maternal-effect and primordial germ cell gene *nanos* was isolated following characterization of the gene. The regulatory elements of this gene will be highly beneficial in constructing a more efficient site-specific recombination system, which should make it easier to achieve various desired transgenic system combinations and generate composite strains of medfly for a better SIT.

1. INTRODUCTION

1.1. The fruit industry and fruit fly problems

The fruit industry is a large and flourishing one as fruits form an important part of the daily diet of people in several continents of the world. Together with vegetables, it is vital to many European countries where both account for about a quarter of total agricultural output (European Commission Directorate-General for Agriculture, 2003). It also provides employment and income to farmers and exporters all over the world, and as noted by Stefan *et al*. (2003) this sector has recorded continuous growth during the past years in developing countries due to the increasing demands in domestic and foreign markets. For example, the Foreign Agricultural Service/USDA Office of Global Analysis (Jan. 2011) in its 2010/2011 citrus world market and trade forecast did forecast that strong demand for orange in the EU and Russia will stimulate additional export from South Africa, Morocco and Turkey. Many European countries have well flourishing and lucrative fruit processing and drink production sectors and these help to meet beverage demand, boost employment and improve their economies. On the other hand, fruit farmers, traders and lastly the economies of the exporting countries invariably profit from the booming trade.

Over the years, tephritid fruit flies have posed a major threat to the fruit industries in several parts of the world. Large and flourishing fruit and vegetable industries have been threatened with collapse while emerging ones have been hindered by the activities of these fruit-infesting flies. Several species of fruit flies are known to be extremely destructive pests of fruits and vegetables (Klassen and Curtis, 2005). This is as a result of the oviposition of eggs into fruits by these flies, and the subsequent tunnelling, eating and damage of the fruits by the larvae emerging from these eggs. Also, microbial infections of the ovipunctures lead to rotting and fruit fall. Yields are lost, fruits become unfit for consumption and fruit marketability becomes highly reduced. Generally, fruit flies are major economic pests because they have a multivoltine life cycle (White and Elson-Harris, 1992), with an explosive reproductive capacity, the capacity to exploit many host plants, the ability to disperse widely as adults or be moved as larvae in fruits and the ability of the adults to survive several months of unfavourable weather (Klassen and Curtis, 2005). Economically important fruit flies in different parts of the world include species from the *Ceratitis, Bactrocera, Ragoletis,*

Anastrepha and *Tephritis* genera. According to Lysandrou (2009), fruit flies like the Mediterranean fruit fly *Ceratitis capitata*, the olive fruit fly *Bactrocera oleae* and the peach fruit fly *B. zonata* have become the notable causative agents of a severe regional pest problem in Arab countries, as well as bringing a barrier to export due to quarantine laws to stop spreading a pest to pest-free countries. As at the late 90s, annual fruit losses to *C. capitata* alone in some countries in the Mediterranean Basin were estimated at about US $365 million (Enkerlin and Mumford, 1997). Elsewhere in countries in Africa and Asia, profitable fruit production is greatly hampered (Lux *et al*., 2003; Vayssieres *et al*., 2005; Stonehouse *et al*., 1998) and free trade in fruits between countries is also in jeopardy due to the presence of fruit flies (Guichard, 2008).

1.2. Fruit fly management and control methods

Various methods have been applied and are still used to control and manage tephritid fruit flies. One of the widely used methods is the application of chemical insecticides (APHIS, 2006; Jessup *et al*., 2007; Gonzalez and Troncoso, 2007; Lysandrou, 2009), though they are of environmental concern and liable to resistance from insects. Biological control measures involving the use of parasitoids, predators and pathogens (Montoya *et al*., 2007; Lux *et al*., 2003) are also applied against fruit flies with varying degree of successes. The Sterile Insect Technique (SIT), which uses released reproductively sterile males to control wild populations, is a strategy that is currently most widely applied for managing tephritid fruit flies (Klassen and Curtis, 2005; Enkerlin,2005). Elimination of male flies through the Male Annihilation Technique (MAT) (Steiner and Lee, 1955; Cunningham, 1989) is another method that is easy to apply and can also achieve considerable success in controlling insect pests. Other methods include cultural practices such as orchard sanitation and fruit bagging (Ekesi and Lux, 2006), protein bait sprays (Mau *et al*., 2007; APHIS, 2006), phototoxic dye food baits (Moreno and Mangan, 2000) and use of traps containing protein baits, pheromones or parapheromones (Lux *et al.*, 2003). To achieve better management and control of fruit flies in infested areas, several control programmes now adopt a strategy termed area-wide integrated pest management (AW-IPM) in which suitable and compatible control methods are combined to manage total pest populations more efficiently across an area of concern.

1.3. The Sterile Insect Technique (SIT) and its improvement

Klassen (2005) called the Sterile Insect Technique (SIT) "a form of birth control imposed on an insect population to reduce its numbers". The principle of SIT is quite simple and according to Knipling (1955), it involves "the release of large numbers of reproductively sterile male insects into a wild population of the same species so that they mate with and block the reproduction of wild females". This method can therefore be applied only against insect species that reproduce by sexual means. Sterility is usually induced by radiation, the method of choice, though chemosterilants were previously tried (Robinson, 2005). SIT is target-specific and environment-friendly (Hendrichs *et al.*, 2002; Wimmer, 2005), and its application should reduce pesticide use.

Operational SIT programmes apply it as one or a combination of the following strategic options: suppression, eradication, containment and prevention. FAO (2005) defined suppression as the application of phytosanitary measures in an infested area to reduce pest population, eradication as the application of phytosanitary measures to eliminate a pest from an area and containment as the application of phytosanitary measures in and around an infested area to prevent the spread of a pest. Prevention is defined as the application of phytosanitary measures in and around a pest-free area to avoid the introduction of a pest (Hendrichs *et al*., 2005).

Developed originally for the New World Screwworm *Cochliomyia hominivorax* by E. F. Knipling (Klassen and Curtis, 2005), SIT has so far become an increasingly important component of AW-IPM programmes for many key insect pest species (Franz, 2005). Over the years, it has been used to prevent, contain, suppress or eradicate fruit fly pests in different parts of the world. These include the eradication and preventive releases of sterile melon flies along the Southern islands of the archipelago in Japan, to avoid re-establishment of the Melon fly, *B. curcubitae* coming from Taiwan (Kuba *et al.*, 1996), the containment, suppression and eradication of the Queensland fruit fly, *B. tryoni* in eastern Australia (Jessup *et al*., 2007), eradication of the Mediterranean fruit fly, *C. capitata* in Southern Mexico (Hendrichs *et al.*, 1983) and several other medfly suppression programmes in Cap Bon, Tunisia (Oritz Moreno, 2001), Hex River, South Africa (Barnes *et al.*, 2004), Madeira, Portugal (Dantas *et al.*, 2004) and Valencia, Spain (Generalitat Valenciana, 2003). Field releases of sterile individuals of *R. cerasi* were conducted in Switzerland for the eradication of this pest (Boller *et al.*, 1975) and

SIT has also been listed among the different control strategies being used to tackle the new peach fruit fly, *B. zonata* in Egypt (Joomaye *et al*., 1999).

To increase the efficiency of fruit fly SIT and improve on the economics and safety of its operation, several transgenic improvements have been made over the years. Notable improvements were in the areas of:

A. Sexing (sex separation) for male-only release

From its basic principle, SIT involves the use of sterile insects to reduce the wild population. Fruit fly SIT is more efficient when only males are released (Rendon *et al*., 2004) as release of only male flies should lead to a higher reduction in the targeted population than a bisexual release because all the released males would then seek wild females instead of getting distracted by co-released sterile females. Beside this, sterile females when released may attempt to oviposit in fruits and create openings which will serve as routes for infections by microorganisms. Sterile flies are usually mass-produced before release and the cost of production of sterile flies would be halved if only males should be mass-produced. However, a sex separation strategy is necessary to produce only male flies for subsequent field releases.

A Genetic Sexing Strain (GSS) was developed using classical genetics for sex separation in the Mediterranean fruit fly *C. capitata* (Franz *et al*., 1994) utilizing a mutant and recessive temperature sensitive lethal (*tsl*) gene to enable separation of males from females by killing of the females. A translocation of the wild-type allele of the *tsl* gene to the Y chromosome makes males heterozygous for this gene while females are homozygous and express only the mutation (Franz 2005). Females die on exposure of embryos to temperatures of 31-35ºC and males survive and are mass-produced. A mutant *white pupae* (*wp*) gene (Rössler, 1979) was also linked to the *tsl* gene and used as a visible marker. Many production facilities presently use the GSS with worldwide production estimated at about 3500 million per week, whilst increasing productions are expected (Franz, 2005). However, the strain has a problem with males having reduced fertility as a result of segregation of unbalanced gametes from the translocations resulting in reduced egg hatch from females and reduced survival of progeny during later developmental stages, in addition to the reduced viability of the females that are

homozygous for the recessive *tsl* mutant gene (Robinson, 2002). In as much as a sexing strain would be desirable for other tephritid pest species, it is difficult to transfer or replicate this GSS in other species. GSS use in operational SIT has so far only been obtained in medfly (Franz, 2005).

Using molecular technologies, transgenic sexing strains (TSSs) were developed to overcome some of the limitations of and possibly replace the classical GSSs. Some of the earlier TSSs were developed in the model fly *Drosophila melanogaster* (Heinrich and Scott, 2000; Thomas *et al*., 2000) based on female elimination via female-specific lethality and used a tetracycline-repressible binary system to make the lethality conditional. Later, bold steps to create a TSS in a tephritid fly were eventually made in medfly using the gene *transformer* (*Cc-tra*), an auto-regulatory gene of the sex-determination cascade in medflies (Pane *et al*., 2002). An RNAi-based system tried to achieve male-only progeny by knocking down *Cc-tra* and transforming females into males, resulting in 95% male and 5% intersex flies (Saccone *et al*., 2007). Fu *et al.* (2007) engineered a transgenic female-specific lethality system in medfly by using the alternatively spliced intron of the sex determination gene *transformer* (*Cctra-I*) (Pane *et al.*, 2002) to regulate and confer dominant lethality (Gong *et al*., 2005) to only female individuals (Fig.1.1). While this system presents a good transgenic alternative to GSS, it showed lethality mostly in pupae. Nevertheless, individuals from TSSs are expected to exhibit better vigour, stability and fertility than their GSS counterparts. Furthermore, a TSS developed in one pest species should be easier to transfer to other closely related pest species compared to GSSs.

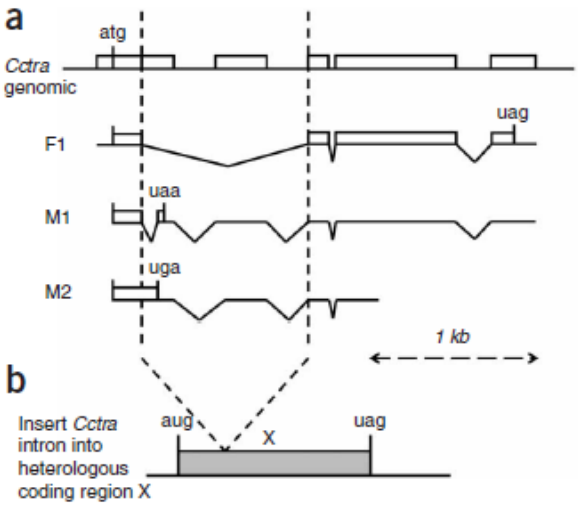

Figure 1.1. Alternative splicing of *Cctra* and its application (Fu *et al.*, 2007)

B. Sterilization of insects prior to release

A crucial factor in SIT is that the released male insects are actually sterile. FAO (2005) defined a sterile insect as "an insect that, as a result of appropriate treatment, is unable to produce viable offspring". It is therefore important that sterile males are still able to perform like the wild-type males (mate, transfer sperms and accessory gland fluids, etc.), albeit incapable of producing viable offspring. This is important for modulating female post-mating responses such as temporal or permanent refractoriness to further mating and change in female behaviour (Robinson, 2005).

The method of choice for inducing sterility in insects intended for release in AW-IPM programmes is now by ionizing radiation (Bakri *et al*., 2005). Cobalt-60 and caesium-137 are commonly used to produce gamma radiation for SIT. Chemosterilants were previously used and though they had efficacies similar to radiation, they are no longer used because of environmental and human-health concerns (Guerra *et al*., 1972; Flint *et al*., 1975; Hayes, 1968; Bracken and Dondale, 1972). Besides, there are also concerns about insects developing resistance to chemosterilants (Klassen and Matsumura, 1966). The radiation dose applied to sterilize an insect is very important to any conventional SIT programme. As radiation negatively affects insects and increase in radiation doses to achieve higher sterility reduces insect competitiveness, the sterilization process is optimized such that both sterility level and competitiveness are balanced to achieve an effective programme (Lance and McInnis, 2005; Bakri *et al*., 2005; Calkins and Parker, 2005). In mass-production facilities across the world, different radiation doses are used for different and even the same fruit fly species (IDIDAS, 2004). Radioactive sources deplete over time, recharge of these sources are expensive and complicated, difficulties exist increasingly with supply of irradiators to some parts of the world (Robinson and Hendrichs, 2005), while radiation safety issues follow usually reported cases by the IAEA of accidental exposure to Co-60 gamma rays (IAEA, 1996; Gonzalez, 1999).

Horn and Wimmer (2003) developed a transgenic strain for insect pest management in *D. melanogaster* which achieved sterility based on a conditional embryonic lethality that kills all offspring of wild females mated to the transgenic males. This system has so far been transferred to the medfly *C. capitata* and the males were found to be highly competitive (Schetelig *et al*., 2009a) (Fig. 1.2). Another transgenic strain developed by Gong *et al*. (2005) also caused sterility by killing of offspring after mating of the transgenic males to wild type

females, but most of the lethality occurs at late larval stage. These transgenic systems offer alternatives with a better safety level than radiation usage. Moreover, issues of accidental releases of untreated insects, which can happen in facilities before insects are irradiated, will not exist any longer. With regards to the economics of production, an embryonic lethality system should lead to mass-production at a reduced cost for an operational programme since released transgenic males should be more competitive than radiation sterilized ones and a lesser number will be needed in the field.

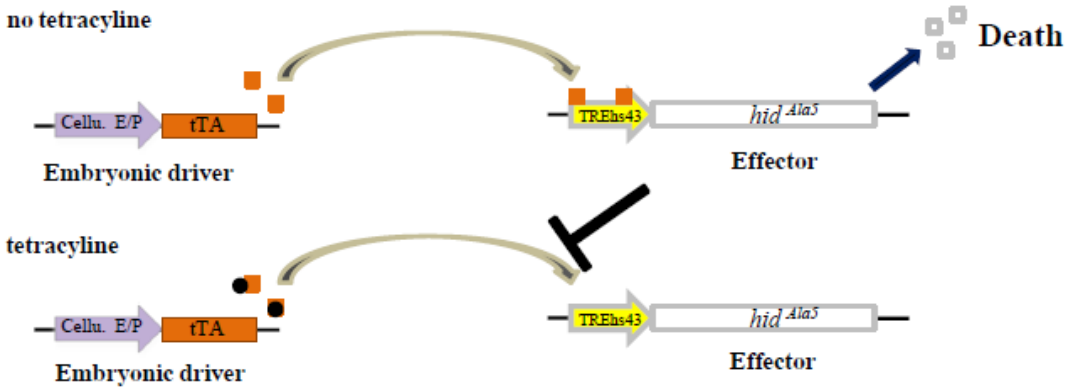

Figure 1.2. Schematic representation of a conditional embryonic lethality system (Horn and Wimmer, 2003; Schetelig *et al.*, 2009a)

C. Monitoring of released insects

Monitoring of both wild type and released insects is an important component of SIT. Sterile insects for release are marked with dyes (Parker, 2005), and traps are usually employed to catch the insects in the field. Data from these field traps reveal the impact of the released insects on the wild population by giving an indication of the number of wild females mating with sterile males, the change in age structure of the target population and the decline in the density of the target population (Vreysen, 2005). The use of dyes is error-prone, labour intensive, expensive and dangerous to human health, while alternatives like morphological markers are associated with loss of competitiveness (Hagler and Jackson, 2001; Robinson and Hendrichs, 2005).

General body marking by fluorescent proteins is a good molecular strategy to identify released flies from wild type flies and this should not have the above mentioned

disadvantages. However, marking of sperms is also desirable and important so as to follow sperm transfer and detect siring efficiency of released males. Marking of insect sperms with a fluorescent protein was done first in the mosquito *Anopheles stephensi* (Catteruccia *et al*., 2005) and later *Aedes aegypti* (Smith *et al*., 2007). This has subsequently been replicated in medfly *C. capitata* where the fluorescence was shown to be detectable even months after death of the flies (Scolari *et al*., 2008). The afore-mentioned systems all utilized the promoter region of the spermatogenesis-specific gene *ß2-tubulin* to drive testis-specific expression of fluorescent proteins and the fluorescent sperms can be detected in the spermathecae of wild females mated to the transgenic males. The males of the medfly sperm-marked strain have also been shown to be highly competitive and generally this approach offers a good, robust, cost-effective and hazard-free alternative for marking insects intended for SIT releases.

1.4. Modification of transgenic strains for SIT

Transgenic technology has so far enabled the engineering of transgenic insects expressing many different traits for use in pest or disease vector control, as well as for research. Transposable elements usually used in generation of transgenic insects are typically non-autonomous and as a result should remain in the same genomic position after insertion. However, one concern exists for transgenic insects designed for use in pest or vector control programmes such as the Sterile Insect Technique (SIT) as this involves inundative field releases of sexually sterile insects to reduce the numbers of wild population of the same species (FAO 2005; Klassen 2005). Many of the transposable elements used in insect transgenesis are insect-derived (Atkinson *et al*., 2007), and therefore the potential presence of transposases required for the "jumping" activities of some of the transposons in the field or elsewhere cannot be argued. Remobilization of a transgene to other genomic locations or even loss of the transgene might occur following contact between a transposase and its specific transposable elements in a transgenic insect. Moreover, a report also exists on cross-mobilization of a *hobo* transposable element by a different but closely related *Hermes* transposase endogenous in the housefly *Musca domestica* (Atkinson *et al*., 1993). These are of ecological concern, and furthermore, is of high importance to programmes like SIT (Schetelig *et al*., 2009b) as a key factor to the success of SIT lies in the insects retaining their qualities pre and post release. It is therefore important that transgenes are made stable in strains intended for SIT applications.

Stabilization of a transgene in an insect genome was first shown in *D. melanogaster* by deletion of transposon vector inverted terminal repeats after genomic integration, and the stabilized transgene was unable to be remobilized in the presence of a transposase source (Handler *et al*., 2004). This strategy was later adopted by Schetelig *et al*. (2009b) in combination with the *phiC31* site-specific recombination system (Thorpe *et al*., 2000), which allows unidirectional site-specific integration of *attachment B* (*attB*)-containing transgenes into an *attachment P* (*attP*) site previously inserted into the genome, to achieve transgene stabilization in the medfly *C. capitata* and created transgenic pest strains for an ecologically safer SIT.

However one looks at it, it clearly appears that biotechnological approaches have the potential to bring far reaching improvements that can shape SIT into a better and more formidable weapon for fighting fruit flies of agricultural importance and other insect pests as well.

1.5. Rationale and objectives of research

A noble transgenic alternative to the genetic sexing strain GSS in the medfly *C. capitata* engineered by Fu *et al*., (2007) using a sex-specifically spliced *Cctra-I* intron (Pane *et al.,* 2002) to confer lethality to only females was shown to have lethality mostly in pupae. An early-acting female-specific lethality system that ensures elimination of food consuming larvae, just as seen in GSSs, would be better for SIT because it should achieve a more cost-effective sex separation and increase mass-rearing efficiency. A good way to get rid of larvae will be to engineer a female-specific lethality system that acts at such an early stage in development as embryogenesis.

Thus, the prime objective of this research was to develop an early-acting transgenic sexing system based on female-specific embryonic lethality in the Mediterranean fruit fly, *C. capitata*. To establish female-specific embryonic lethality and create a male-only strain, an embryonic lethality system (Horn and Wimmer, 2003; Schetelig *et al*., 2009a) was combined with a female-specific lethality system employing the sex-specifically spliced *Cctra-I* intron (Fu *et al*., 2007).

Once a male-only strain had been generated and proven to function satisfactorily, the *phiC31* site-specific recombination system (Thorpe *et al*., 2000; Groth *et al*., 2004) was utilized to modify the transgene in the genome of the lines and stabilize it against subsequent remobilization (Handler *et al*., 2004; Schetelig *et al*., 2009b). Strains possessing stabilized transgenes should thereafter be suitable and ready for potential field use in SIT programmes for the control of medfly.

Because the present site-specific integration strategy in *C. capitata* which involved co-injection of a desired plasmid and capped *phiC31* integrase mRNA (Bischof *et al*., 2007; Schetelig *et al*., 2009b) might not be so efficient to achieve all the desired site-specific integrations, the development of a more efficient strategy is needed. Such a high-efficiency strategy for site-specific integration of transgenes into genomic locations using the *phiC31* site-specific recombination system was developed for *D. melanogaster* using endogenous promoters of maternal-effect and primordial germ cell genes (Bischof *et al*., 2007). Such a gene, *nanos*, from *C. capitata* has therefore been isolated and characterized, and its regulatory region obtained so that it can subsequently be utilized to develop a *phiC31* site-specific recombination strategy that should be of better efficiency than what presently exists for this fly.

2. MATERIALS AND METHODS

2.1. Medfly strains

Wild type *Egypt II* and *we,wp* strains were obtained from the FAO/IAEA Agriculture and Biotechnology Laboratory (Entomology Unit, Seiberdorf, Austria) and maintained under standard rearing conditions in the laboratory (Saul, 1982).

2.2. Cloning of sexing effector plasmid construct, #1402

A 940 bp *attP-TREhs43* and 1.3 kb *Cctra-I* intron fragments were amplified by PCR from the plasmid constructs #1247 (*pBac* [*attP-TREhs43hidAla5_Pub-EGFP*]) (Schetelig *et al.*, 2009a) and #1301 (*pBac* [*attP-sryα2-Cctra-tTA_Pub-DsRed*]) (Schetelig *et al.*, 2011) respectively using the primers mfs 309/310 (mfs309-ATCCGCGGACTAGGGTGCCCCAACTGG; mfs310-GTAGGTCTCTACCATTGTGTGGGTG) and mfs 300/306 (mfs 300 - GTAGGTCTCATGGTAATTTTAAAAGCATATTTTTTTCTTTGAAATTC; mfs 306 - AGTAGGCCTATAGATACCATAGATGTATGGATTAG). Both fragments were cut by *SacII*/*Eco31I* and *Eco31I/StuI* respectively and cloned together by ligating their *Eco31I* cut ends. The *attP-TREhs43-Cctra* fragment was inserted into *SacII/StuI*-cut pSL_fa1180fa vector to create #1400 (pSL_fa_*attP-TREhs43-Cctra*_fa). The 4 kb fragment *hid*^{Ala5}-*SV40polyA* was amplified from #1247 (Schetelig *et al.*, 2009a) using primers mfs307/308 (mfs307-CCGTGCCCTTTTATTTGCCCGAG; mfs308-GAAGCTTGAGCTCGAGATCTAGGC), cut by *BglII* and inserted into *StuI/BglII*-cut #1400 to create #1401 (pSL_fa_ *attP-TREhs43-Cctra-hid*^{Ala5}*-SV40polyA*_fa). Finally, the fragment *attP-TREhs43-Cctra-hid*^{Ala5}*-SV40polyA* was excised out of #1401 via *AscI* digest and inserted into *AscI*-cut #1201 (pBac *fa_Pub-nls-EGFP-SV40 polyA*) to generate the sexing effector plasmid construct #1402 (pBac *fa_attP-TREhs43-Cctra-hid*^{Ala5}*-SV40polyA_a_Pub-nls-EGFP-SV40 polyA*).

2.3. Medfly germ line transformation

Microinjection of wild type embryos was done as previously described (Schetelig *et al.*, 2009a) using construct #1402 (500 ng/µl) and *piggyBac* (*pBac*) helper plasmid *phspBac* (Handler and Harrell, 1999) (200 ng/µl). Out of 708 injected embryos, 240 hatched larvae were collated, 92 survived to pupae and 78 successfully emerged as adults. Each male G0 adults was out-crossed to 4 WT virgin females, while each G0 female was out-crossed to 2 WT males. G1 adult flies were screened for *EFGP* expression by epiflourescence microscopy using the fluorescence stereomicroscope LEICA MZ16 FA with the filter EYFP (Ext. 500/20; Emm.535/30). Expression of *EGFP* confirms the presence of the sexing effector construct. The different heterozygous lines (showing different fluorescence patterns) were inbred and homozygous individuals selected by their increased level of green fluorescence. The transgenic fly lines were maintained under standard rearing conditions (Saul, 1982).

2.4. Lethal strains and female-specific lethality assessments

Lethal strains were generated by crossing adult flies from the sexing effector lines with those from the driver lines, *sl1-tTA*, *sryα2-tTA*_f2m1, *sryα2-tTA*_f4m1, *sryα2-tTA*_m2m1 and *CG2186-tTA* (Schetelig *et al.*, 2009a). Homozygous flies from the sexing effector lines were crossed with homozygous flies from driver lines to generate double heterozygous lethal strains. The double heterozygous lethal strains were inbred and their progeny screened to select only individuals homozygous for both the driver and sexing effector construct (double homozygous) by epifluorescence microscopy based on fluorescence intensity. Flies in all crossings were maintained on 10 µg/ml tetracycline adult diet.

To assess female-specific lethality in double heterozygous condition, homozygous adults from a driver line were crossed to homozygous adults from a sexing effector line and kept on tetracycline-free adult diet. The adult progeny carrying the driver and sexing effector constructs in a double heterozygous condition, were separated according to sex, screened for both EGFP and DsRed expression and counted. To assess female-specific lethality in the double homozygous condition, flies from the double homozygous lethal strains were simply raised on tetracycline-free adult diet and the sex ratio of their progeny determined.

2.5. Lethality stage tests

Double homozygous flies from the lethal strain #43B (*sryα2-tTA*_m2m1 x 1402_22m1B) were kept on tetracycline-free adult food. Eggs were collected from them 24 hours after egg-laying onto petri dishes containing tetracycline-free larval diet in three replicates and counted under a binocular microscope. Larval hatches from the eggs were scored about 24 hours after egg collection. The petri dishes were observed daily and the numbers of dead larvae were scored. The petri dishes were cross-checked for any larvae (dead or alive) left behind 4 days after pupation. Pupae were sieved out from sand, counted and kept in Styrofoam-closed plastic vials. Emerging adults were separated according to sex and scored. Another experiment was carried out in parallel with strain #43B flies reared on 10 µg/ml tetracycline.

2.6. Reverse Transcriptase PCR (RT-PCR)

Total RNA was isolated from 0-48 hour old embryos of lethal strains kept on tetracycline-free medium using TRIzol reagent (Invitrogen, Carlsbad CA). cDNA was reverse transcribed from total RNA using First Strand cDNA Synthesis Kit (Fermentas Life Sciences, EU) according to manufacturer's instructions. The primer pair of CO1/CO18 (CO1-ACAAGAAGAGCAAAGTGCCA; CO18-TCTGCTGGCTCTGGTACATCCTG) was used to amplify all possible splice variants while CO1/CO7 (CO1-ACAAGAAGAGCAAAGTGCCA; CO7-TTTTCCCTTTCCTTGGTTTACTTA) was used to amplify only the male-specific splice variants.

2.7. *In situ* hybridization

All DIG-labelled RNA probes were prepared using the DIG-RNA-Labelling Kit (Roche, Mannheim).

2.7.1. *In situ* hybridization on FSEL #43B embryos

The primers mfs300 (GTAGGTCTCATGGTAATTTTAAAAGCATATTTTTTTCTTTGAAATTC) and CO7

(TTTTCCCTTTCCTTGGTTTACTTA) were used to amplify an 807 bp *Cctra-I* fragment which was cloned into pCRII (Invitrogen, Karlsruhe), linearized with *BamHI* and transcribed *in vitro* with T7 RNA polymerase to generate the antisense *Cctra-I* RNA probe. Whole mount *in situ* hybridization (WMISH) of antisense *tTA*, *hid*^{Ala5} (Schetelig *et al.*, 2009a) and *Cctra-I* RNA probes to 0-48 hrs old embryos was then done as described (Davis *et al*. 2001).

2.7.2. *In situ* hybridization on WT ovaries and embryos

In vitro transcriptions were performed on plasmid *Ccnos-pCRII*_3 (see section 2.15) linearized with *BamHI* and transcribed with T7 RNA polymerase to generate the antisense probe, and the same plasmid linearized with *EcoRV* and transcribed with Sp6 RNA polymerase to generate the sense probe.

Newly eclosed female flies were kept on adult diet, anaesthetized using CO_2 after 24-60hrs and their abdomens were cut off. Ovaries were dissected out from the cut abdomen in 1X PBS and immediately fixed in a solution of 1:1 fixation buffer (3.7% formaldehyde in PBS) and heptane for 2 hrs on a rotator. The ovaries were washed three times in PBS, dehydrated stepwise in methanol and stored at – 20ºC. Subsequently, ovarioles were dissected from the ovaries in PBS, treated with 20µg/mL proteinase K at 37ºC for 30min and rinsed in PBT for 5min. The dissected ovarioles were then post-fixed in a solution of 3.7% formaldehyde in PBT for 30min and washed six times in PBT. Whole Mount *in Situ* hybridizations of sense and antisense *nos* probes respectively to the ovarioles were performed as described in Davis *et al*. (2001).

Embryos (0-48hrs old) were fixed and hybridizations of sense and antisense *nos* probes respectively to the embryos done as described (Davis *et al*., 2001).

2.8. Molecular characterization of sexing effector lines

2.8.1. Inverse PCR on genomic DNA from sexing effector lines

Inverse PCR was performed for three potent sexing effector lines to identify genomic DNA sequences flanking *pBac* insertions in them. About 1.25µg genomic DNA was digested with *MspI* for 24hrs, ethanol precipitated and self-ligated in a final reaction volume of 500µl at

14ºC for about 1½ days. PCRs on the self-ligated genomic DNA from the different lines was performed as described (Horn *et al*., 2003), purified and sequenced directly using the amplification primers together with primers mfs227 (GTACTGTCATCTGATGTACCAGGCACTTCATTTGGC) included for the 3´junction and co29 (TCGTTGTGTTGCTCTGTGATC) included only for the 5´junction of line 1402_21m2. Obtained sequences are available in appendix 2.1.

2.8.2. PCR to confirm presence of *attachment P* (*attP*) sites

Prior to co-injection of sexing effector lines with plasmid #1252 (Schetelig *et al.*, 2009b) and capped *phiC31* mRNA, PCRs were performed on genomic DNA from the three potent lines #1402_21m2, #1402_22m1A and #1402_22m1B using primers mfs373 (ATCTTGACCTTGCCACAGAGGACTATTAGAG) and mfs360 (GCCTAGCGACCCTACGCCCCCAACTGAG) to confirm the presence of the 51bp *attP* site embedded in the transgenes they carry. PCR product sequencing revealed good integrity of *attP* site (appendix 2.2).

2.9. Site-specific integration in sexing effector lines

In vitro transcription of capped *phiC31* integrase mRNA from the *BamHI*-linearized plasmid *pcDNA3.1-phiC31* (Bischof *et al*., 2007) was performed using the mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX) according to manufacturer's instruction. To achieve site-specific integration, the plasmid construct #1252 (Schetelig *et al.*, 2009b) was co-injected with capped *phiC31* integrase mRNA (200ng/µl DNA: 600ng/µl RNA) into embryos of sexing effector lines: 761 embryos from #1402_21m2, 229 embryos from #1402_22m1A and 85 embryos from #1402_22m1B. The G0 adults were out-crossed to WT flies of the opposite sex and the resulting G1 adult progeny were screened for red and green body fluorescence using the fluorescence stereomicroscope LEICA MZ16 FA with the filter DsRed (Ext. 545/30; Emm. 620/60) and EYFP (Ext. 500/20; Emm. 535/30) respectively. Expression of DsRed fluorescence indicates occurrence of integration of plasmid #1252 into the germ line of the sexing effector line, while the original green fluorescence pattern confirms the background

of a line. Heterozygous individuals showing both red were inbred and homozygous individuals selected based on their increased fluorescence intensity.

2.10. Transgene remobilization

To remobilize transgenes in the integrated line #1402_22m1B_int, homozygous individuals were crossed with those from *Jump_3* line and then out-crossed to individuals from *we,wp* strain as described (Schetelig *et al*., 2009b). Individuals that had supposedly successful remobilization events were selected based on their expression of only a green fluorescence pattern similar to that of the original line #1402_22m1B (in the mouthparts, thorax and legs) and white eyes which indicate absence of *pBac* transposase source.

2.11. Molecular characterization of integrated and stabilized lines

To confirm site-specific integration in the integrated line #1402_22m1B_int, PCR was performed on its genomic DNA using the following primers as described (Schetelig *et al*., 2009b): mfs373 (ATCTTGACCTTGCCACAGAGGACTATTAGAG) and mfs372 (TTGAGCTCGAGATCTGTGAGGTGGAGTACG) to amplify the *attR* junction; mfs 330 (GCTCATCGACTTGATATTGTCCGACAC) and mfs360 (GCCTAGCGACCCTACGCCCCCAACTGAG) to amplify the *attL* junction. PCR products were cloned into pJET1.2 vector (Fermentas Life Science, EU) and then sequenced. Also primers mfs373 and 360 were used for PCR to check for the presence of a free *attP* site (fig. 2.3A).

Because the original line #1402_22m1B carries two copies of the same transgene inserted by *pBac* transposition in two different genomic locations, PCR was performed first on genomic DNA from wild type adults using primers co38 (GGTCCATATCGTTAATCAAACTCAA), co39 (GGAGTATTGACTGTTATGGCACCTAA), co40 (CATAACGAGAACGTACCCAACAGT) and co41 (GATTCCATGTAACCCAGGCGTC) that bind the genomic DNA sequences immediately flanking the *pBac* transposon ends. This allowed matching of the primers binding adjacent wild type genomic DNA sequences.

To find out which transgene received site-specific integration, PCR was performed on genomic DNA from the integrated line using primer combinations co38/mfs372 and co39/mfs372 respectively (fig. 2.2B & 2.3B). Following transgene remobilization, lines having the correct Inverted Terminal Repeat (ITRs) excision were determined by PCR on their genomic DNA using primers co39 and co33 (GACCCTACGCCCCCAACTGAGA) (figure 2.5A). The amplified products were cloned into *pJET1.2* (Fermentas Life Science, EU) and sequenced. Loss of the 2nd transgene lacking integration was determined using primers co38/33 and co38/40 (fig. 2.5B & C). The presence or absence of any unmodified transgene in other genomic locations in the stabilized lines was checked using primers mfs373/360 which should amplify the *attP* site that is part of the *pBac*-inserted #1402 in the original sexing effector line.

2.12. Test for stability

The stability of the transgenes in line #1402_22m1B_stab_f4 was assessed by crossing 10 homozygous males of this line with 10 homozygous females of the *Jump_3* line. From the resulting progeny, 24 females were selected and out-crossed with 24 *we,wp* males. All the progeny from egg collections made on the last out-crossing were screened using the fluorescence stereomicroscope LEICA MZ16 FA with the filter EYFP (Ext. 500/20; Emm.535/30) for the old and any new green fluorescence patterns. The same experiment was performed in parallel with individuals from the original potent sexing effector line #1402_22m1B.

2.13. Test for female-specific lethality in stabilized line

To assess female-specific lethality in the stabilized line #1402_22m1B_stab_f4, 10 males from this line were crossed with 10 females from the embryonic driver line #1244_m2m1 and kept on adult diet lacking tetracycline. Double heterozygous progeny from the crossing were separated according to sex and scored.

A double homozygous lethal strain was generated by crossing stabilized line #1402_22m1B_stab_f4 individuals with those from the embryonic driver line #1244_m2m1 and raising them on adult diet containing 10µg/ml tetracycline. The double heterozygous

adults were then inbred on food containing same concentration of tetracycline and the double homozygous ones were selected and raised separately. Female-specific lethality in double homozygous condition was then subsequently conducted by raising some double homozygous individuals on tetracycline-free food and scoring the sex of their progeny at adult stage.

2.14. Cloning of *attB*-sexing effector plasmid construct, #1408

Plasmid construct #1250 (Schetelig *et al*., 2009b), containing one 3'*pBac* end and an *attachment site* (*attB*), was digested with *ApaI* and *EcoRI* respectively to remove the 3'*pBac* end and generate a linear plasmid. The ends of the linear plasmid were blunted using T4 DNA polymerase (Fermentas Life Sciences, EU) and then ligated together to generate plasmid #1404 (pSL_*attB*). A 672bp 5'*pBac* end was amplified by PCR using primer co10 (CTAGGCCGGCCAACCCTAGAAAGATA) and primer co11 (CCAAAGCTTGACAATGTTCAGTGCA), digested with *HindIII* and inserted into the *StuI*/*HindIII*-cut #1404 to create plasmid construct #1405 (pSL_*attB*_5'*pBac*). A 2.98kb *PUbDsRed* marker cassette (Handler and Harrell, 2001) was amplified from plasmid #1252 (Schetelig *et al*., 2009b) by PCR using primer co8 (CTATCCGGATCTCGAGCTCAAG) and primer co9 (ATAGGCCGGCCAGATACATTGATGAGT), digested with *BspMII*/*FseI* and inserted into the *BspMII*-cut and *FseI* partially digested #1405 to create plasmid construct #1406 (pSL_*attB_PUbDsRed*_5'*pBac*). Partial digestion of #1405 with *FseI* was performed because this plasmid contains three *FseI* restriction sites one of which was needed for insertion of the *PUbDsRed* marker cassette. Primer co30 (AGGAGATCTAGGCCGGCCGAATTTCGAGT) and primer 31 (CGCTCCGGACTTGGTCGAGCTGATACTTC) were used for the PCR amplification of the 6.2 kb sexing effector cassette (*TREhs43-Cctra-hid*^{Ala5}*-SV40polyA*) from plasmid #1402 (see section 2.2). The amplified sexing effector fragment was digested with *BglII*/*BspMII* and inserted into *BglII*/*BspMII* cut #1406 to generate plasmid construct #1408 (pSL_*attB*_ *TREhs43-Cctra-hid*^{Ala5}*-SV40polyA*_ *PUbDsRed*_5'*pBac*).

2.15. Isolation and cloning of *C. capitata nanos* (*Ccnos*) cDNA and genomic DNA

Searches for the nanos protein sequences from three cyclorrapha dipterans, *D. melanogaster*, *D. virilis* and *Musca domestica* were performed and obtained using NCBI protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome). Based on alignments of the most conserved regions of the three proteins, degenerate primers were made that should bind DNA sequences coding for the amino acids HCVFCENN and KPIITMED respectively. The degenerate primers nosF1 (CAYTGYGTNTTYTGYGARAAYAAY) and nosR1 (RTCYTCCATNGTDATDATNGGYTT) were used to amplify a *nos* fragment from cDNA pool as described (Schetelig *et al*. 2008a). The amplified product was cloned into pCRII vector (Invitrogen, Karlsruhe), sequenced and a protein BLAST was performed using the amino acid sequence obtained after translating the DNA sequence. Following recognition of the clone *nos*-pCRII_3 containing the degenerate primer-amplified conserved *nos* fragment as a homolog of *Dmnos*, gene-specific primers were made using the sequence from the insert in this plasmid. The gene-specific primers nos-GSP_1 (ACTGTATGCGCCTTATCCCCCGAG) and nos-GSP_2 (AACGAACCGGAAGCTGTAGTGAGAAGTC) were used to isolate the 5´ and 3´ ends of *Ccnos* respectively as described (Schetelig *et al*., 2008a) using the Clontech SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). RACE products were cloned into pCRII vector (Invitrogen, Karlsruhe), sequenced and put together to compile a *Ccnos* full cDNA sequence *in silico*. Primers nos5'UTR_F (TAAATTCTTTCAGTAAGTTGGAAGCA) and nos3'UTR_R (AGATAAGTTTGAATACTCTTTATTTATTTACA) binding the ends of the two untranslated regions of *Ccnos* respectively were used to amplify a 5.8kb fragment from genomic DNA and this was subsequently sequenced. All amplifications were done using Advantage 2 polymerase (Clontech, Mountain View, CA) except the final amplification of full length genomic DNA region of *Ccnos* which was done using the proof-reading Phusion polymerase (New England Biolabs).

2.16. Gene analysis and reconstruction

Sequence obtained following sequencing of products from PCRs on cDNA or genomic DNA was analyzed and translated using VectorNTI Advance 10 (Invitrogen). Analysis of translated

protein was done using NCBI protein BLAST. Alignment of the conserved regions of CcNOS with NOS proteins of the three cyclorrhapha dipterans mentioned was done using ClustalW2 (http://simgene.com/ClustalW). Positions of introns and exons in genomic DNA were determined by matching the cDNA and genomic DNA sequences using VectorNTI 10 (Invitrogen).

2.17. Inverse PCR on genomic DNA from WT fly to isolate *nanos* upstream sequences

Inverse PCR was performed using genomic DNA from wild type *Egypt II* strain to identify genomic DNA sequences upstream of *Ccnos*. About 800ng genomic DNA was digested with *BglII* at 37ºC for 6hrs, ethanol precipitated and self-ligated in a final reaction volume of 500µl at 14ºC overnight. PCRs on the self-ligated genomic DNA was performed as described (Horn *et al*., 2003) using primers co75 and co76 (AGAGATCCAAATTCAAGAAAAGAAACGGC and CCTAGTCTTTCGCCTACATCTACGGAATCG), gel-extracted, purified, cloned into *pCRII* vector (Invitrogen, Karlsruhe) and sequenced.

3. RESULTS

3.1. Establishment of a transgenic female-specific embryonic lethality system in the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae)

Described here is a female-specific embryonic lethality system established for sex separation in the medfly *C. capitata* as a transgenic alternative to the classical Genetic Sexing Strain (GSS).

3.1.1. Construction of a female-specific embryonic lethality system

Construction of a female-specific embryonic lethality (FSEL) system was done using a tetracycline-repressible binary expression system (Gossen and Bujard, 1992). Since the intention was to restrict lethality to only females, the alternatively spliced intron of the sex-determination gene *tra-I* of *C. capitata* (*Cctra-I*) (Pane *et al.,* 2002) was employed as it had earlier been used to engineer female-specific lethality also in *C. capitata* (Fu *et al.,* 2007). The female-specific embryonic lethality system was designed such that: (i) the driver construct is composed of the heterologous transactivator gene *tTA* placed under the control of promoter/enhancer of a cellularization-specifically expressed gene (fig. 3.1.1A) to ensure blastoderm-specific expression of tTA (Horn and Wimmer, 2003; Schetelig *et al.,* 2009a). In this study, the same driver lines used by Schetelig *et al.* (2009a) in achieving embryonic lethality in *C. capitata* were used. These driver lines have *tTA* placed under the control of different cellularization-specific promoter/enhancers namely *Cc-serendipity* α (*Cc-sryα*), *Cc-slow as molasses* (*Cc-slam*) and *Cc-CG2186*; (ii) the sexing effector construct, which contains the *D. melanogaster* pro-apoptotic gene *Dm-hid*^{Ala5} under the control of the *tTA-response element* (*TRE*), was designed to carry the *Cc transformer-I* intron (*Cctra-I*) to confer female-specific functionality of *Dm-hid*^{Ala5}. For the insertion of *Cctra-I* into *Dm-hid*^{Ala5}, the first intention was to choose such a position that it would be immediately flanked by the dinucleotides TG and GT supposedly important for correct splicing (Fu *et al.,* 2007), but such a position exists only far down in the coding region of *hid*^{Ala5} that this might lead to a partially functional HID^{ALA5}. Therefore, the *Cctra-I* intron was placed immediately behind the ATG of

Dm-hid^{Ala5} (fig. 3.1.1B) in a position where it is flanked by the dinucleotides TG and GC. This was cloned into a plasmid bearing both 5' and 3' *piggyBac* (*pBac*) ends and *PUb-EGFP* (Handler and Harrell, 1999) to create a sexing effector plasmid construct, #1402 (fig 3.1.1B). The expectation on a sexing effector designed this way was that after its successful integration into the genome, the *Cctra-I* in its transcripts will be completely spliced out only in females leading to correct translation of *Dm-hid*^{Ala5}, while the stop codons in the male-specific transcripts will stop the complete translation of *Dm-hid*^{Ala5} in males (fig 3.1.2A). A combination of both components of the described system should lead to early embryonic expression of *tTA* in both sexes, which subsequently activates the sex-specifically spliced effector. After correct splicing of transcripts, complete translation of *Dm-hid*^{Ala5} only in females should lead to the death of all female embryos. The lethality should be suppressible by tetracycline supplied maternally to embryos by adding it to the adult diet (Bello *et al.*, 1998; Schetelig *et al*., 2009a). In addition, both components of the lethality system carry an *attachment P* site (*attP*) (fig. 3.1.1A & B) which will allow for future site-specific modification of the genome-integrated transgene (Venken *et al.*, 2006; Schetelig *et al.*, 2009b).

3.1.2. Sex-specific and tetracycline-repressible lethality

Plasmid #1402 (fig. 3.1.1B) was used to create sexing effector lines by *pBac* mediated transposition. Lines #1402_21m1, #1402_21m2 and #1402_22m1 were generated. The lines were identified by epifluorescence and each line had a specific fluorescence pattern different from others. To assess the three sexing effector lines for mediation of female-specific lethality, homozygous flies from these lines were crossed with homozygous flies from a set of five different driver lines, *sl1-tTA*, *sryα2-tTA*_f2m1, *sryα2-tTA*_f4m1, *sryα2-tTA*_m2m1 and *CG2186-tTA* (table 1.1, Schetelig *et al*., 2009a) resulting in the generation of double heterozygous FSEL strains. FSEL strain #32 showed about 72% reduction of females, while FSEL strains #31 and #52 showed 47% and 42% female reduction respectively (table 1.1). Lethality in both males and females was shown by FSEL strains #33, #43 and #53, with strains #33 and #43 both having 100% lethality. Other FSEL strains showed little or no female reduction.

All FSEL strains were also assessed for level of lethality in flies carrying double copies of both driver and sexing effector (double homozygous individuals) as this has been shown to increase the efficiency of lethality (Heinrich and Scott, 2000) and would also resemble a factory rearing situation. For a FSEL strain like #32 which showed high female reduction (table 1.1), it is expected that double copies of each construct should lead to higher expression of their products and therefore to stronger female-specific lethality. Double homozygous condition for FSEL strain #32 eventually yielded 100% removal of female progeny. Surprisingly, homozygous conditions for both transgenes in FSEL strain #42 led to almost complete female removal; up from non-detectable female-specific lethality in double heterozygous conditions to 99% males in the double homozygous condition (table 1.1). FSEL strains #31 and #41 also showed high female-specific lethality leading to about 95% and 92% male progeny respectively. In FSEL strains #33, #43 and #53, double homozygous condition led to complete lethality in both males and females. Double homozygous condition also increased female-specific lethality in other FSEL strains, but they were below 90% male progeny (table 1.1). The high female-specific lethality showed by the FSEL strains #31, #32, #41 and #42 indicate that the two driver lines *sryα2-tTA*_f4m1 and *sryα2-tTA*_m2m1 which were used to generate these strains are strong drivers.

The behaviour of the sexing effector line #1402_22m1 when crossed to the driver lines *sryα2-tTA*_f4m1, *sryα2-tTA*_m2m1 or *CG2186-tTA* to generate FSEL strains #33, #43 or #53 that showed lethality in both male and female progeny in the absence of tetracycline (table 1.1) was not expected. To further examine this sexing effector line, remobilization of the sexing effector transgene in this line to different genomic positions was undertaken. #1402_22m1 flies were crossed with those from a jump starter line *Jump_3* (Schetelig *et al*., 2009b) carrying the transposase needed for *pBac* transposition. Successful remobilization of the sexing effector transgene resulted in the generation of three additional sexing effector lines #1402_22m1A, #1402_22m1B and #1402_22m1C, also identified by their new fluorescence patterns different from other lines. Homozygous flies from these three additional sexing effector lines were respectively crossed with those from the driver lines *sryα2-tTA*_f4m1 or *sryα2-tTA*_m2m1 to generate FSEL strains and assessed for female-specific lethality. The driver lines *sryα2-tTA*_f4m1 and *sryα2-tTA*_m2m1 were chosen because they had been shown to mediate strong expression of *tTA* in *C. capitata* embryos (Schetelig *et al*., 2009a) and had also proved to be strong drivers for female-specific lethality (table 1.1). Interestingly, the FSEL strains #33A and #33B generated by crossing the driver line *sryα2-tTA*_f4m1 to the

sexing effector lines #1402_22m1A and #1402_22m1B, respectively, showed very high levels of female-specific lethality (99-100% males) in double heterozygous progeny, while the FSEL strain #33C generated by crossing the driver line *sryα2-tTA*_f4m1 to the sexing effector line #1402_22m1C had a weaker level of female-specific lethality (table 1.1). FSEL strain #43A that was generated by crossing sexing effector line #1402_22m1A to driver line *sryα2-tTA*_m2m1 showed 94% males, while FSEL strain #43B which was generated by crossing sexing effector line #1402_22m1B to driver line *sryα2-tTA*_m2m1 showed complete removal of females in the double heterozygous condition. In the double homozygous condition, FSEL strains #33A, #33B and #43B showed complete elimination of females, but strain #33C could not achieve removal of all females (table 1.1). No assessment could be made on double homozygous individuals of FSEL strain #43A since such a strain could not be generated. Inbreeding of the double heterozygous #43A individuals on several trials failed to yield any double homozygous progeny. One possible explanation for this could be that both the driver and sexing effector transgenes are on the same or close locations in the genome thereby making it impossible to have double copies of each transgene. In general, the best female-specific lethality was that recorded in FSEL strain #43B which showed 100% female-specific lethality in both double heterozygous and double homozygous adult progeny, with a total of 8,315 double homozygous individuals screened (table 1.1, appendix 1B). The above result showed that genomic integration sites or "position effects" also exert an influence on splicing and can make a splicing-based system highly penetrant, weak or to even function improperly. In addition, it lends support to the modulation of transgene expressions by genomic integration sites (Schetelig *et al*., 2009a; Schetelig *et al*., 2011).

Repression of female-specific lethality by tetracycline was assessed using the FSEL strain #43B since it exhibited the strongest level of female-specific lethality (table 1.1). 10 µg/ml of tetracycline in parental adult and larval diet was able to repress female-specific lethality in double homozygous progeny of this strain. Tetracycline repression test on double heterozygous strain of #43B was not performed since actual sex separation during mass-production for SIT applications should take place on double homozygous strains.

3.1.3. Female-specific lethality acts during embryogenesis

As the initial intention was to create female-specific embryonic lethality, the period when female-specific lethality takes place in FSEL strains was checked. Embryos from the FSEL strain #43B were taken and their development was followed to the adult stage. For progeny from parents kept on tetracycline-free diet, only a small percentage of the progeny died in the 1st larval instar stage within 24 hours after hatching (table 1.2). No lethality was observed during the 2nd and 3rd larval instar stages or in pupae, while the emerging adults were all males indicating that female-specific lethality must have occurred during embryogenesis. There were few larval deaths and an almost equal ratio of emerging male and female adult progeny for both #43B parents kept on 10µg/ml tetracycline diet and wild type. Whole mount *in situ* hybridizations (WMISH) performed on embryos from strain #43B kept on tetracycline-free diet indeed showed a sequential activation of components of the female-specific embryonic lethality system during embryogenesis. Strong *tTA* expression was observed during cellularization (fig. 3.1.3B). *Dm-hid*^{Ala5} and the *Cctra-I* intron within its coding region were weakly detected during cellularization (fig. 3.1.3F & J) and strongly detected during germ band elongation (fig. 3.1.3G & K) and germ band retraction (fig. 3.1.3H & L). The strong staining of *Cctra-I* intron within the coding region of *hid*^{Ala5} in #43B embryos is in contrast to wild type embryos where hybridization with the same *Cctra-I* intron RNA probe failed to give any detectable signal (not shown).

3.1.4. Adequate protection of males from splicing-regulated lethality

An important attribute of this lethality system should be the strict female-specificity of its lethality, which in principle should have no effect on male individuals carrying copies of the lethal transgene combination. Since the FSEL strains #33, #43 and #53 carrying the sexing effector line #1402_22m1 showed lethality both in male and female progeny in double heterozygous and double homozygous condition when reared without tetracycline (table 1.1), some of the sexing effector lines derived from it (which interestingly mediated highly penetrant female-specific lethality when crossed to strong driver lines) were examined to determine how well the males are protected from splicing-regulated lethality. This was done by comparing the number of male flies that were double heterozygous for the transgene combination to those that were heterozygous for one transgene. Genotypic status of

heterozygosity or homozygosity was determined by epifluorescence microscopy based on fluorescence intensity. Homozygous flies from the driver line *sryα2-tTA*_m2m1 were crossed with heterozygous individuals of the opposite sex from the sexing effector line #1402_22m1B. Male progeny having the transgene combination were not significantly lesser than those having only the driver construct (table 1.3A), indicating that they are adequately protected and that the lethality is strictly female-specific in the double heterozygous conditions. Male protection from lethality was also checked in double homozygous condition by inbreeding double heterozygous parents and rearing them on tetracycline-free diet. The number of double homozygous male adult progeny compared well to the number of wild type males and fits well with the classical Mendelian ratio (table 1.3B), indicating also that they are well protected from lethality. PCRs on cDNA from #43B embryos were performed with one primer, CO1, binding the 5'UTR of *Dm-hid*^{Ala5} and another primer, CO18, binding complementarily in the coding region of *Dm-hid*^{Ala5}, or alternatively primer, CO7, binding complementarily in the male specific region of the *Cctra-I* (fig. 3.1.2A). Fragments corresponding to the predicted F1, M1 and M2 splice variants (418bp, 723bp & 805bp) for the first primer combination and only the male-specific splice variants M1 and M2 (257bp & 340bp) for the second primer combination were obtained (fig. 3.1.2B). This supports the notion that male survival is due to male-specific splice variants with stop codons in the sexing effector transcript which prevents organismal death in males. Also present were fragments corresponding to the predicted unspliced transcripts for the first and second primer combinations (1763bp and 888bp respectively). Some fragments that are supposedly partially spliced transcripts (Pane *et al*., 2002) were also present for both primer combinations.

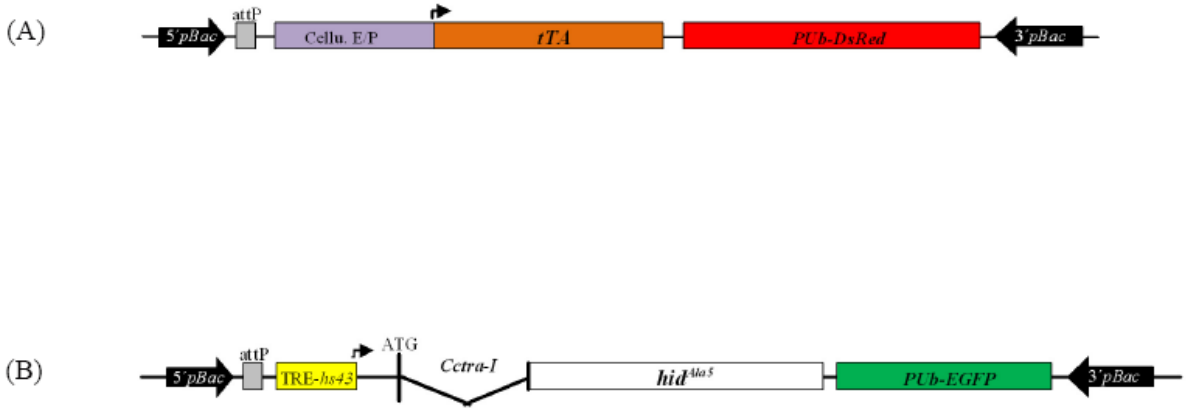

Figure 3.1.1. Binary expression system for female-specific embryonic lethality. (A) Promoters/enhancers of cellularization genes limit the expression of *tTA* to early embryonic stages. tTA should bind to the tetracycline-response element (TRE) in the sexing effector plasmid construct #1402 (B) and then drives the expression of downstream genes. *Cctransformer-I* intron (*Cctra-I)* is sex-specifically spliced within the coding region of *Dm-hid*^{Ala5} and restricts HID-induced lethality to only female individuals.

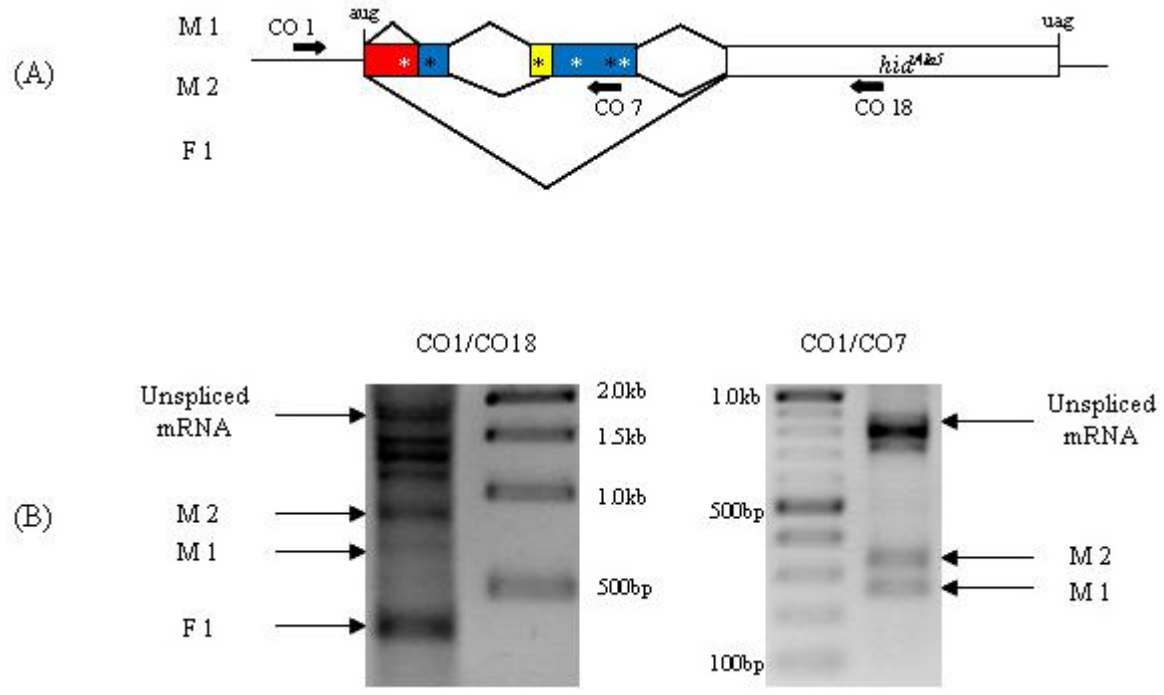

Figure 3.1.2. Sex-specific splicing of *Cctra-I* intron. (A) The expected sex-specific alternative splicing of *Cctra-I* intron within the coding region of hid^{Ala5}. Expression of hid^{Ala5} and the consequential HID-induced lethality is expected only in females where the whole *Cctra-I* intron (F1) is spliced out. The stop codons in the male splice variants (M1 & M2) should protect males from HID-induced lethality. Black asterisk indicate the stop codons in M1 splice variant and white asterisk indicate stop codons in M2 splice variants. (B) Sex-specific splicing checked by PCR on cDNA product from #43B embryos (0-48hrs). PCR using primers CO1/CO18 yielded the predicted female-specific splice variant (F1, 418bp) and male-specific splice variants (M1, 723bp & M2, 805bp) respectively, while primers CO1/CO7 yielded only the predicted male-specific splice variants (M1, 257bp & M2, 340bp). Also present are fragments corresponding to the predicted unspliced mRNA (1763bp in COI/CO18; 888bp in CO1/CO7) and supposedly partially spliced mRNA.

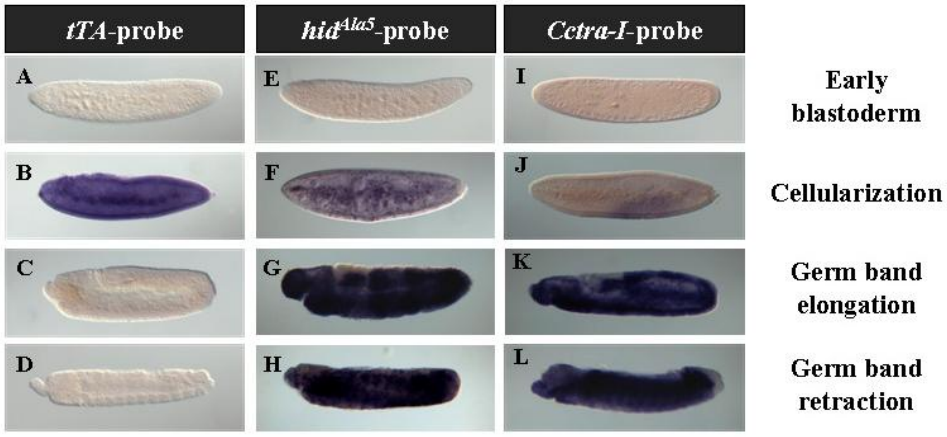

Figure 3.1.3. Activation of components of female-specific embryonic lethality system. Whole mount *in situ* hybridization of antisense RNA probes to embryos of strain #43B (0-48hrs) show the absence of *tTA* mRNA during early blastoderm (A), detection of its expression during cellularization (B) and non-detection during germ band elongation/retraction (C & D). tTA subsequently drives the expression of hid^{Ala5} and the *Cctra-I* intron within the coding region of hid^{Ala5}. mRNAs of both hid^{Ala5} and *Cctra-I* intron become weakly detectable during cellularization (F & J) and are very strongly expressed during germ band elongation and retraction (G & H, K & L).

Table 1.1. Assessment of FSEL strains for female-specific lethality

Sexing effector	Driver	FSEL strain	Tet.	Double het. progeny ♂:♀ (%)	Double homo. progeny ♂:♀ (%)
1402_21m1	*srya2-tTA*_f2m1	#21	-	53.9:46.1	64.2:35.8
1402_21m1	*srya2-tTA*_f4m1	#31	-	65.5:34.5	94.9:5.1
1402_21m1	*srya2-tTA*_m2m1	#41	-	52.0:48.0	91.6:8.4
1402_21m1	*CG2186-tTA*	#51	-	51.7:48.3	76.3:23.7
1402_21m2	*srya2-tTA*_f2m1	#22	-	54.5:45.5	69.1:30.9
1402_21m2	*srya2-tTA*_f4m1	**#32**	-	78.1:21.9	100:0.0
1402_21m2	*srya2-tTA*_m2m1	#42	-	47.3.0:52.7	99.1:0.9
1402_21m2	*CG2186-tTA*	#52	-	63.9:36.1	85.2:14.8
1402_22m1	*sl1-tTA*	#13	-	52.1:47.9	62.6:37.4
1402_22m1	*srya2-tTA*_f2m1	#23	-	51.4:48.6	53.8:46.2
1402_22m1	*srya2-tTA*_f4m1	#33	-	0.0:0.0	0.0:0.0
1402_22m1	*srya2-tTA*_m2m1	#43	-	0.0:0.0	0.0:0.0
1402_22m1	*CG2186-tTA*	#53	-	0.0-55.2:0.0-44.8	0.0:0.0
1402_22m1A	*srya2-tTA*_f4m1	**#33A**	-	99.4-100:0.0-0.6	100:0.0
1402_22m1A	*srya2-tTA*_m2m1	#43A	-	94.0:6.0	*
1402_22m1B	*srya2-tTA*_f4m1	**#33B**	-	99.0-100:0.0-1.0	100:0.0
1402_22m1B	*srya2-tTA*_m2m1	**#43B**	- +	100:0.0 NA	100:0.0 49.0:51.0
1402_22m1C	*srya2-tTA*_f4m1	#33C	-	53.4:46.6	66.5:33.5

- indicates no tetracycline in diet and + indicates 10µg/ml tetracycline in adult and larval diet. #43B was used for tetracycline suppression test because it showed the strongest female-specific lethality. Suppression test was conducted using only double homozygous strains, since actual sexing in rearing facilities should be on double homozygotes and not on double heterozygotes (NA). In bold letters are FSEL strains that showed complete removal of females. *Double homozygous condition for this transgene combination could not be established as a strain.

Table 1.2. Stage of female-specific lethality

Developmental Stage	Wild Type			#43B					
				10µg/ml Tet. diet			Tet.-free diet		
Eggs	305	285	293	292	315	245	233	225	292
Hatched Larvae (L1)	160	98	84	172	141	67	94	83	92
Dead L1	0	1	4	1	0	1	3	11	8
Dead L2	1	1	0	0	1	0	0	0	0
Dead L3	0	0	0	0	0	0	0	0	0
Pupae	152	93	78	168	138	61	91	72	83
Male Adults	64	53	37	65	61	27	87	67	72
Female Adults	74	35	33	69	58	25	0	0	0

Female-specific lethality at the embryonic stage in progeny from strain #43B parents reared on tetracycline-free diet is deduced from the larval mortality that was not very different from those on tetracycline and the wild type control and then the final absence of female adults.

Table 1.3. Protection of males from lethality

A. Double heterozygous progeny

Transgenes	Males	Females
Driver only (red fluorescence)	708	624
Driver & Sexing Effector (red & green fluorescence)	705	0

Homozygous flies from the driver line *sryα2*_m2m1 were crossed with heterozygous flies from the sexing effector line 1402_22m1B. The resulting progeny carry one copy of the driver construct or one copy each of both the driver and sexing effector constructs. The sexing effector line 1402_22m1B was particularly chosen because it showed the highest level of female-specific lethality when crossed to driver lines in previous tests.

B. Double homozygous progeny

Transgenes	Males	Females
Wild type	121	131
Driver & Sexing Effector (double homozygous)	122	0
Other phenotypes together	1675	437

Double heterozygous flies from #43B were inbred and a total of 2486 (1918♂s and 568♀s) of the resulting progeny adults were screened and their genotypes identified by fluorescence. The number of surviving double homozygous males when compared to wild type males showed that males do not suffer from lethality. #43B was used because it showed the highest level of female-specific lethality in previous tests.

3.2. Site-specific recombination to stabilize evaluated and potent transgenic lines of the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae) for SIT use

Described here is the successful stabilization of the evaluated and most potent sexing effector line used to generate the male-only strain. The *phiC31* site-specific recombination system was employed to achieve site-specific integration of a transgene carrying an additional Inverted Terminal Repeat (ITR) into an attachment P (*attP*) site in this line, while post-integrational transgene stabilization was achieved by deleting the transgene part between the 5'*pBac* ITR and the first 3'*pBac* ITR (see fig 2.1) leaving only one ITR (3'*pBac*) that can no longer mediate remobilization.

3.2.1. Potent transgenic lines and site-specific integration into their *attP* sites

In the female-specific embryonic lethality system described earlier (see section 3.1), three sexing effector lines were able to achieve complete elimination of double homozygous female progeny when adults were crossed to those from embryonic drivers and raised on food without tetracycline. These three lines, #1402_21m2, #1402_22m1A and #1402_22m1B were therefore selected for stabilization. To start off work with these lines, they were first molecularly characterized. By inverse PCR, the genomic sequences flanking the *pBac* ITRs used for genomic insertion of the transgenes in the lines were identified (appendix 2.1). Line #1402_22m1B was found to contain two insertions of transgene #1402 in its genome (appendix 2.1). The presence and integrity of the 51bp *attP* sequence embedded in the genome-inserted transgene in the three lines was confirmed by PCR (appendix 2.2).

To achieve site-specific integration, plasmid construct #1252 (Schetelig *et al*., 2009b) was co-injected with capped *phiC31* integrase mRNA from *pcDNA3.1-phiC31* (Bischof *et al*., 2007) into embryos from the three mentioned sexing effector lines carrying the plasmid construct #1402 (fig. 3.2.1). Plasmid construct #1252 bears a 64bp *attachment B* (*attB*) site, a red fluorescent marker and a 3´*piggyBac* (*pBac*) end that should enable transgene remobilization following site-specific integration. Successful integration of plasmid #1252 into an *attP* site was obtained only in the sexing effector line #1402_22m1B, and was verified by the red fluorescence pattern which was similar to the parental green fluorescence pattern given the

close genomic location and thus same position effects of both fluorescent markers (fig. 3.2.2B).

3.2.2. Characterization of integrated line

Following the phenotypic identification of an integration event, characterization of the integrated line #1402_22m1B_int was undertaken to molecularly confirm the site-specific integration event. The molecular characterization indeed confirmed a site-specific integration of plasmid construct #1252 into the *attP* site of #1402 with the amplification of perfect *attR* and *attL* junctions (appendix 2.3), but also revealed the presence of a free *attP* sequence suggesting a transgene without integration (fig. 3.2.3A). Since the parent strain #1402_22m1B carries two insertions of the transgene construct #1402 (appendix 2.1), the characterization of integrated line #1402_22m1B_int was extended to also find out which of the two transgene insertions had integration. PCR analysis (see section 2.11) showed that site-specific integration was only in one transgene, thereafter named transgene 1 (figs. 3.2.2B & 3.2.3B; appendix 2.4).

3.2.3. Transgene remobilization and verification of desired *pBac* ITRs excision

To remobilize transgenes in the integrated line #1402_22m1B_int, flies from this line were crossed with individuals from a jumpstarter line *Jump_3* (Schetelig *et al*., 2009b), the F1 from them out crossed to *we,wp* flies and the resulting F2 progeny screened for the loss of red fluorescence. Since the integrated construct #1252 carried a 3´*pBac* end, provision of *pBac* transposase by *Jump_3* should lead to the excision of the single 5´*pBac* end and the first 3´*pBac* end or the single 5´*pBac* end and the second 3´*pBac* end in transgene 1 (figs 3.2.1 & 3.2.2B). Loss of transgene 2 (transgene without integration) from the genome was desired and also expected. Screening of F2 individuals revealed many phenotypes indicating different remobilization events (fig. 3.2.4), but only individuals that have lost the red fluorescence, have the original green fluorescence pattern and also white eyes which signify absence of the transposase source were selected and raised as distinct lines for further analysis.

Post-remobilization characterization to identify successfully stabilized lines was done using primers, co39 and co33, that bind the upstream genomic sequence flanking transgene 1 and

the *attL* junction respectively (fig. 3.2.2C). Three out of 10 lines analyzed (#1402_22m1B_stab m4, m6A and f4) were found to have the correct excision of ITRs expected from transgene 1 (fig 3.2.5A; appendix 2.5). Further analysis by PCR with primers binding genomic flanking sequences (see section 2.11) showed that transgene 2 was removed from its previous genomic position in all the lines (fig. 3.2.5B & C), and by PCR with primers detecting intact *attP* sites that two of the three lines with the correct transgene 1 modification (m4 and f4) had no other unmodified transgene while the third line (m6A) had at least one unmodified transgene somewhere in its genome (fig. 3.2.5D).

3.2.4. Stability test on stabilized line

A hallmark of stable transgenes is immobility on exposure to the corresponding transposase source. To assess for transgene stability, one of the supposedly stabilized lines #1402_22m1B_stab_f4 was crossed with individuals from the jumpstarter line *Jump_3*, their F1 progeny out-crossed to wild type flies and the F2 progeny were screened. #1402_22m1B_stab_f4 was chosen as it had clearer gel bands from PCR characterization than #1402_22m1B_stab_m4 (fig. 3.2.5C). The same experiment was carried out in parallel with individuals from the original line #1402_22m1B. While no new fluorescence pattern was observed in F2 progeny of #1402_22m1B_stab_f4 individuals crossed to those from *Jump_3*, several new fluorescent patterns were observed in F2 progeny of the original line #1402_22m1B (table 2.1). This confirmed transgene stability in the line #1402_22m1B_stab_f4, as instability would have resulted in transgene remobilization and new fluorescent patterns as recorded in the original line #1402_22m1B.

3.2.5. Recheck of female-specific lethality in stabilized line

The key important trait expressed by the sexing effector line #1402_22m1B is the ability to mediate female-specific organismal death in progeny when crossed to a driver line. Because this line was very potent and had two transgene insertions, there was concern over its potency following removal of one of the transgenes during stabilization. To address this, a female-specific lethality test was conducted on the stabilized line #1402_22m1B_stab_f4 by crossing them to those from the embryonic driver line #1244_m2m1 (Schetelig *et al*., 2009a). In the

absence of tetracycline, about 99% female elimination was recorded in double heterozygous progeny while 100% female elimination was observed in progeny having the double homozygous condition (table 2.2). Moreover, female-specific lethality was repressed by the presence of 10µg/ml tetracycline in adult diet.

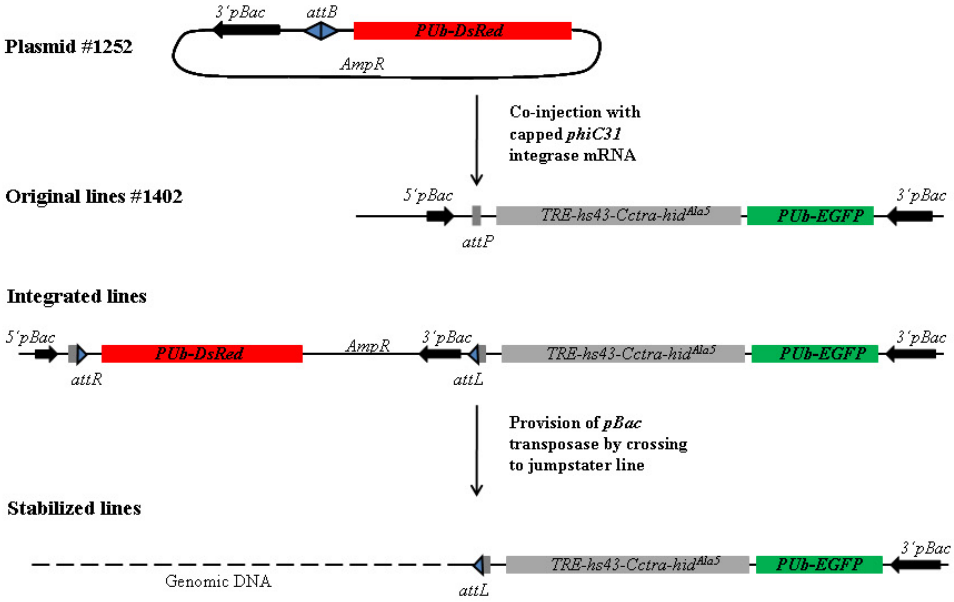

Figure 3.2.1. Strategy to generate a stabilized transgenic sexing effector line (adapted from Schetelig *et al*., 2009b)**.** Co-injection of plasmid construct #1252 and capped *phiC31* integrase mRNA should lead to site-specific integration of the construct into the *attP* site of transgene #1402 which was randomly inserted into the genome of a wild type fly using a *pBac* transposon vector. Provision of *pBac* transposase to the integrated strain could cause remobilization of a part of the transgene (5´*pBac* and 1st 3´*pBac* ends) to leave behind a stable transgene (or series of other transposition events not depicted).

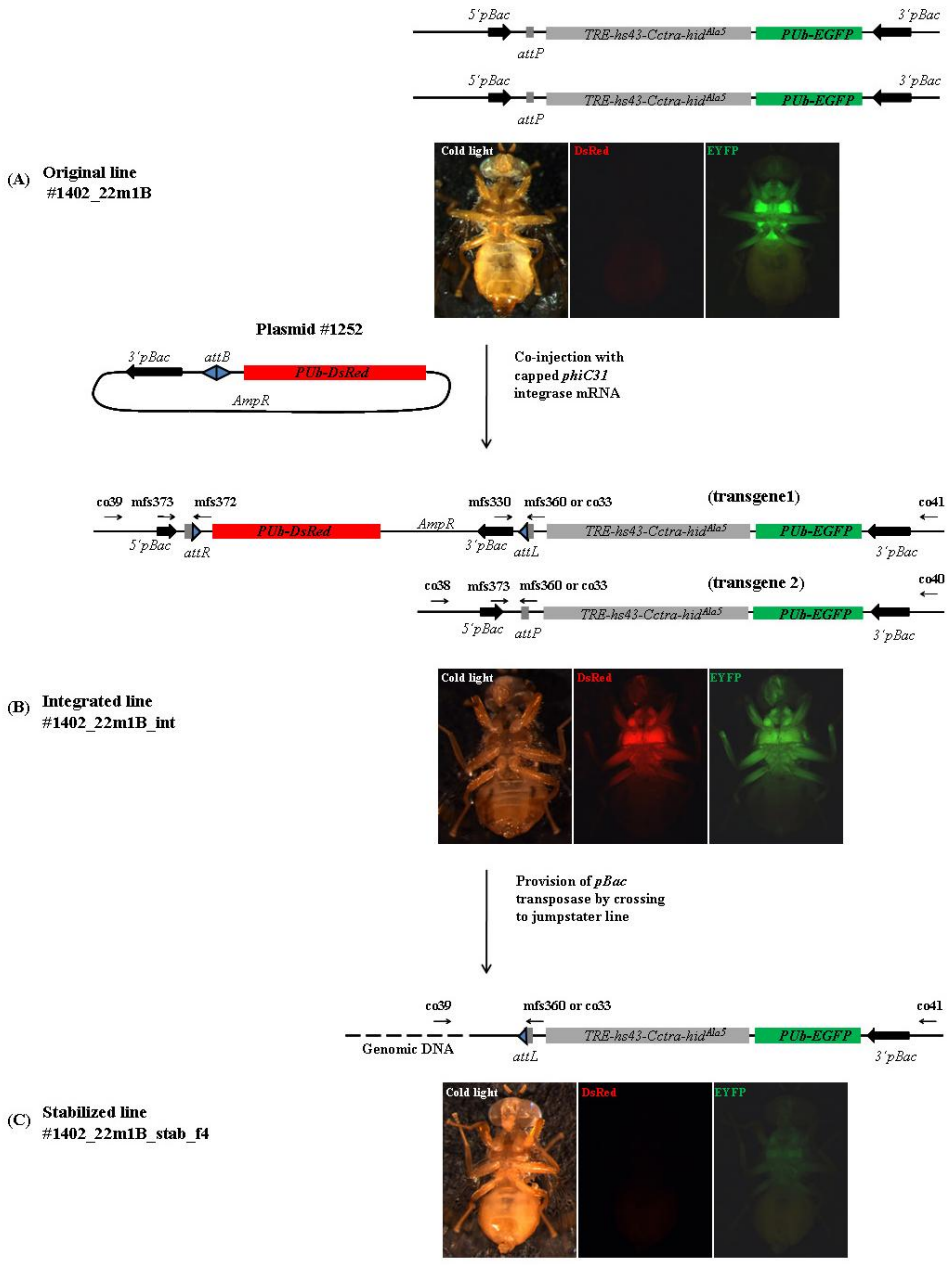

Figure 3.2.2. Generation of the stabilized line #1402_22m1B_stab_f4. The original line #1402_22m1B has two copies of the same trangene inserted by random *pBac* transposition into its genome (A). Co-injection of the original line with plasmid construct #1252 and capped *phiC31* integrase led to site-specific integration of #1252 into the *attP* site in one of the two transgene insertions in #1402_22m1B (B). Provision of *pBac* transposase to the integrated line #1402_22m1B_int by crossing it to jumpstarter line *Jump_3* achieved the desired remobilization event concurrently with loss of the 2nd transgene insertion thereby leading to the emergence of stabilized line #1402_22m1B_stab_f4 (C). Arrows on the transgenes show positions of primers used in characterizing the lines.

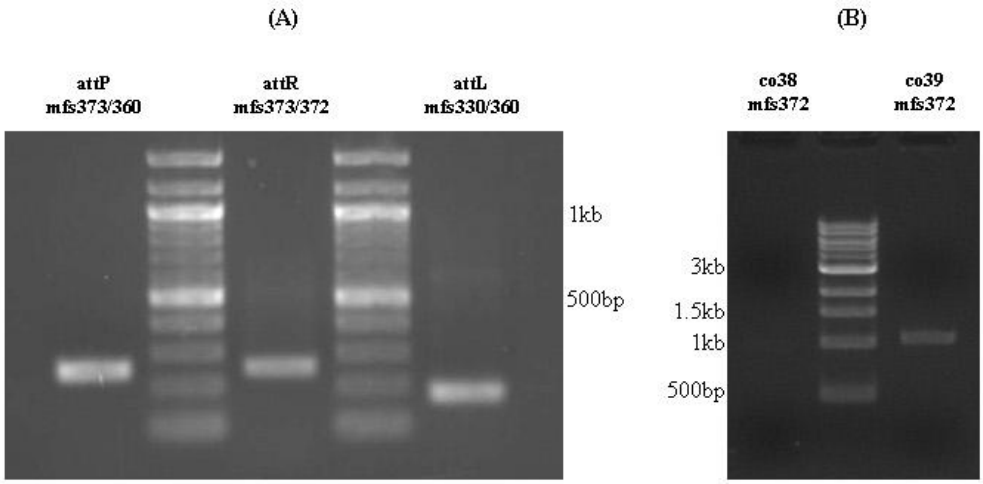

Figure 3.2.3. Analysis on integrated line #1402_22m1B_int. (A) Amplification of fragments of the correct sizes for the *attR* (260bp) and *attL* (185bp) junctions respectively provided support to the phenotypic evidence of site-specific integration in the strain. The amplification of a fragment of the correct size using primers mfs373/360 indicates a free *attP* site (see fig 2.2) and suggests a transgene without integration. (B) The correct fragment (1080bp) got with the primer combination co39/mfs372 shows which of the two transgenes had site-specific integration (see fig. 2.2).

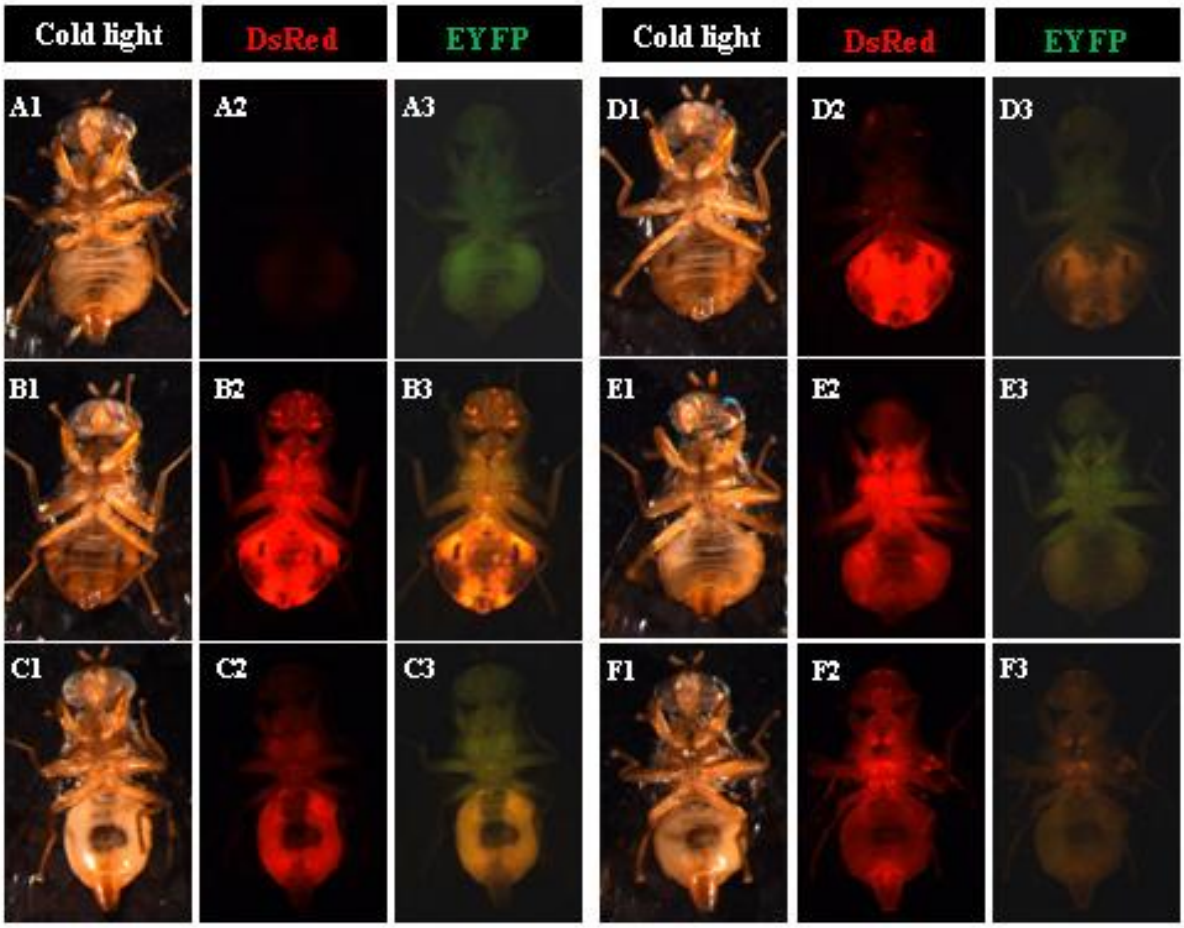

Figure 3.2.4. Other transgene remobilization events. Besides the desired remobilization event shown in fig. 3.2.2C, crossing of the integrated line #1402_22m1B_int to *Jump_3* individuals also led to a series of other remobilization events. Depicted are flies showing the loss of red fluorescence and green fluorescence in the head, thorax, abdomen and legs (A1-3), red head, thoracic, abdominal and leg fluorescence with green thoracic fluorescence (B1-3), red head dots, thoracic, abdominal and leg fluorescence with green head, thoracic and leg fluorescence (C1-3), red head dots, thoracic, abdominal and leg fluorescence with green thoracic and leg fluorescence (D1-3), red and green fluorescence in the head, thorax, abdomen and legs (E1-3), and red head, thoracic, abdominal, leg and wing vein fluorescence with green abdominal fluorescence (F1-3).

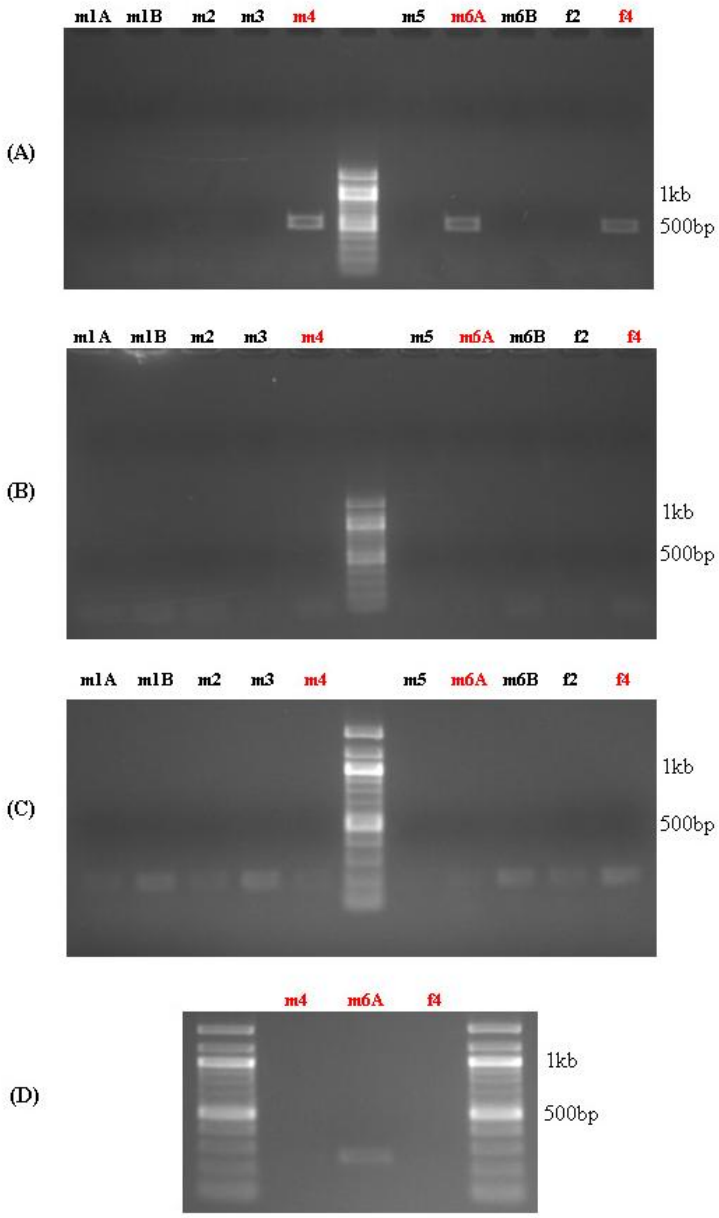

Figure 3.2.5. Characterization of remobilization events. (A) Analysis of 10 remobilization lines using primers co39/33 (see fig 2.2) reveals correct *pBac* ITRs excision in 3 of the lines (m4, f4 & m6A, in red) by the amplification of fragments of the predicted sizes (529bp). Loss of the 2nd transgene insertion was confirmed using primers co38/33 (see fig 2.2) which had no band amplified (B) and primers co38/40 which amplified fragments about the predicted sizes (C). Amplifications of part of transgene #1402 using primers mfs373/360 (see fig 2.2) show that one of the three stabilized lines, m6A, also has at least an unmodified transgene in another genomic position whereas two of the lines, m4 and f4, are free of unmodified transgenes (D).

Table 2.1. Stability test on stabilized line #1402_22m1B_stab_f4

(#1402_22m1B x *Jump_3*) x *we,wp*				(#1402_22m1B_stab_f4 x *Jump_3*) x *we,wp*			
Phenotype	Replicate I	Replicate II	Replicate III	Phenotype	Replicate I	Replicate II	Replicate III
Original fluorescent pattern (MTL strong)	18	19	14	Original fluorescent pattern (MTL moderate)	293	273	306
New fluorescent patterns				New fluorescent pattern	0	0	0
(i) MTL moderate	129	153	162				
(ii) HTLA	17	4	13				
(iii) T	32	44	23				
(iv) TA	1	0	0				
Wild type	216	252	271	Wild type	313	294	379

The absence of individuals with new fluorescent patterns in the F2 progeny of stabilized line #1402_22m1B_stab_f4 crossed to *Jump_3* and out-crossed to *we,wp* shows that this line has a stable transgene which cannot be remobilized, unlike those from the original line #1402_22m1B which has an unstable transgene. Letters signify body part with fluorescence: head (H), mouthparts (M), thorax (T), legs (L) and abdomen (A).

Table 2.2. Female-specific lethality test on stabilized line #1402_22m1B_stab_f4

Adults	#1402_22m1B stab f4 x #1244_m2m1								
	Tc-free, double heterozygous progeny			Tc-free, double homozygous progeny			10µg/ml Tc, double homozygous progeny		
	I	II	III	I	II	III	I	II	III
Males	93	42	86	230	293	262	60	64	60
Females	1	0	1	0	0	0	44	56	58

The stabilized line #1402_22m1B_stab_f4 retained almost the same level of potency after stabilization as the original line #1402_22m1B as shown by its ability to efficiently mediate elimination of all female progeny when crossed to embryonic driver line #1244_m2m1. Tc stands for tetracycline in adult diet.

3.3. Development of an optimized transgenic strain of the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae) by site-specific genomic integration of genes-of-interest into evaluated lines

Described here is an on-going work on the development of an optimized transgenic strain of the Mediterranean fruit fly *Ceratitis capitata* by using *phiC31*-mediated site-specific recombination to integrate desired genes-of-interest (GOI) into specific genomic locations in already evaluated and fit lines, and subsequently achieve cassette exchange and/or transgene stabilization in these lines.

3.3.1. Evaluated and fit transgenic medfly lines

A conditional embryonic lethality system (Schetelig *et al*., 2009a) was developed in medfly using cellularization-specific promoters to regulate a heterologous transactivator *tTA* and drive a pro-apoptotic gene hid^{Ala5}(see section 1.3B, fig 1.2). Lethal strains #67 and #66 from this system have undergone extensive evaluation and performed satisfactorily: the transgenes are well expressed and can be made double homozygous, the strain showed 100% embryonic lethality, the males were found to be fit and highly competitive when compared to wild type, and both molecular and cytogenetic characterization were already done and revealed the genomic flanking sequences and the exact chromosomal locations of the transgenes (Schetelig *et al.*, 2009a). The two strains, #67 and #66, were derived by crossing effector line #1247_f1m2 to embryonic driver lines #1244_m2m1 and #1244_f4m1 respectively (Schetelig *et al.*, 2009a). The transgene in effector line #1247_f1m2 was successfully made stable following site-specific integration and subsequent deletion of part of the transgene between the 5'*pBac* and the first 3'*pBac* ends (Schetelig *et al.*, 2009b; see also fig 3.21). It is desirable to make use of the same genomic insertion sites of transgenes in the evaluated strains #67 and #66 for engineering new medfly strains as this, most probably, will lead to fit and highly performing transgenic medfly strains for SIT applications. The *phiC31* site-specific transgene integration system (Thorpe *et al*., 2000; Groth *et al*., 2004) is one good way to achieve this goal.

3.3.1. Exchange of an effector cassette with a sexing effector cassette and achieve transgene stabilization in an evaluated effector line

An early-acting transgenic sexing strain was established for medfly in this study (see section 3.1). One of the sexing effector lines (#1402_22m1B) used to generate the sexing strain has been stabilized by *phiC31*-mediated site-specific integration and subsequent deletion of part of the transgene between the 5'*pBac* and the first 3'*pBac* ends (see section 3.2, fig. 3.2.2). But the early-acting sexing strains are yet to undergo extensive evaluation to ascertain their level of fitness and competitiveness in comparison to the embryonic lethal strains #67 and #66. At the current absence of such an evaluation, an additional strategy was devised for the exchange of the effector gene cassette in line #1247_f1m2 with a sexing effector cassette so as to generate a sexing effector line that should have the same transgene genomic location (and most probably similar position effects) as the effector line for strain #67 and #66 (#1247_f1m2) (fig. 3.3.1). Plasmid construct #1408 was generated for this purpose, and co-injection of this plasmid with *phiC31* capped integrase mRNA into #1247_f1m2 embryos should lead to integration into the *attP* site embedded in the transgene of line #1247_f1m2 (fig 3.3.1). Remobilization should lead to loss of the effector gene cassette by deletion of part of the transgene between the second 5'*pBac* and the 3'*pBac* ends and leave behind an exchanged-stable sexing effector line (fig 3.3.1).

3.3.2. Addition of a sperm-marking cassette and stabilization of evaluated embryonic driver lines

Generation of an early-acting sexing strain required the combination of a sexing effector and an embryonic driver line. As transgene stabilization of one of the sexing effector lines has been achieved, it is important to also stabilize the transgene in an embryonic driver line to enable the creation of a fully stabilized FSEL strain. The embryonic driver lines #1244_m2m1 and #1244_f4m1 (Schetelig *et al*., 2009a) were selected for transgene stabilization as they generated very good lethal strains when crossed to the potent sexing effector lines (see table 1.1 of section 3.1).

A fluorescent sperm marking system (Scolari *et al.*, 2008) developed for medfly offers a convenient, cost-effective and safer way of monitoring males released in such a programme as SIT. In addition, this system enables the assessment of sperm transfer, sperm competition and

siring capabilities of released males. Development of a strain that integrates this fluorescent sperm marking system with an early-acting sexing system will be most desired for operational SIT as this should offer cost-effective production of only males in facilities and improved field monitoring of released males. The site-specific integration strategy again fits well into the scheme to achieve this objective.

Therefore, plasmid construct #1306 (fig 3.3.2.; provided by Marc Schetelig) was designed to carry the sperm marking gene cassette and also one 3'*pBac* end, an *attB* site and a *PUb-EGFP* marker cassette (Handler and Harrell, 1999) in a way that its successful integration into the *attP* site in the genome inserted embryonic driver transgene #1244 should be in the orientation that will allow for subsequent remobilization to delete part of the transgene between the 5'*pBac* and the first 3'*pBac* ends and leave behind a sperm marked embryonic driver transgene (fig 3.3.2).

Once stable sexing effector and sperm marked embryonic driver lines have been generated, they can be crossed to create an optimized medfly strain for highly improved, cost-effective and ecologically safer SIT.

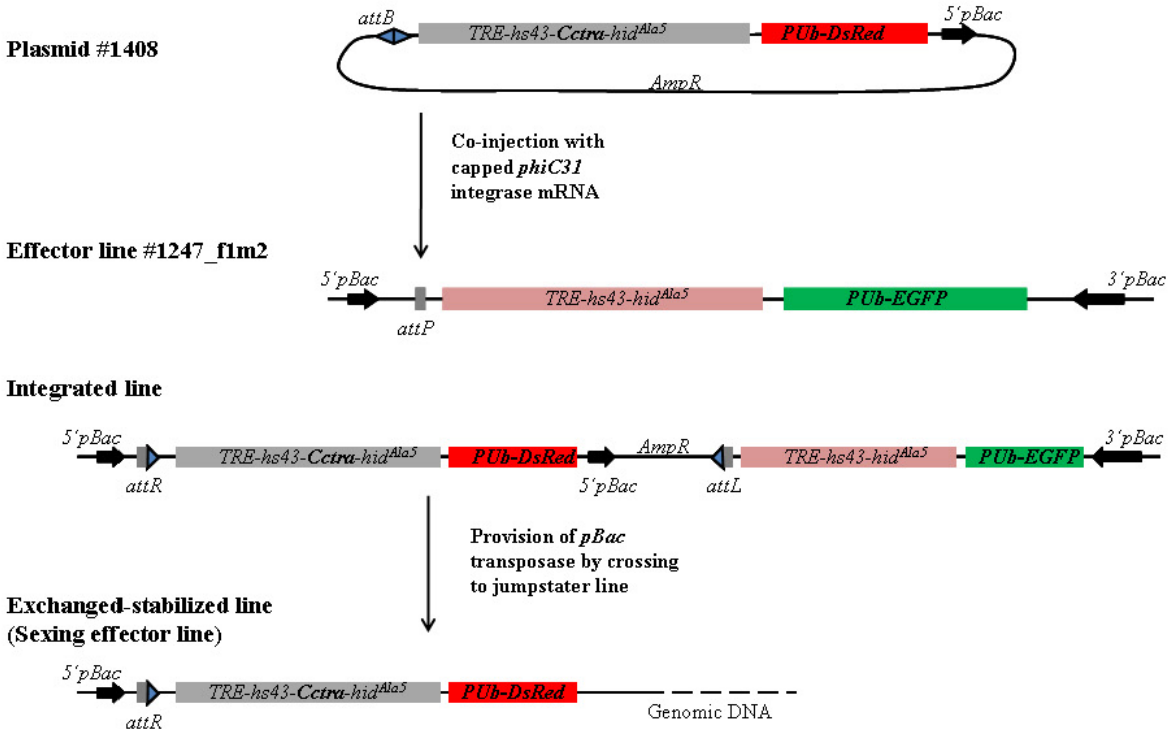

Figure 3.3.1. Strategy to exchange an effector cassette with a sexing effector cassette and achieve transgene stabilization in evaluated effector line, #1247_f1m2. Co-injection of plasmid construct #1408 (containing a sexing effector cassette) and capped *phiC31* integrase mRNA should lead to site-specific integration of the construct into the *attP* site of the transgene #1247 which was randomly inserted into the genome of a wild type fly using a *pBac* transposon vector and resulted in effector line #1247_f1m2. Provision of *pBac* transposase to the integrated line could cause remobilization of a part of the transgene (second 5´*pBac* and 3´*pBac* ends) to leave behind a stable sexing effector transgene (or series of other transposition events not depicted).

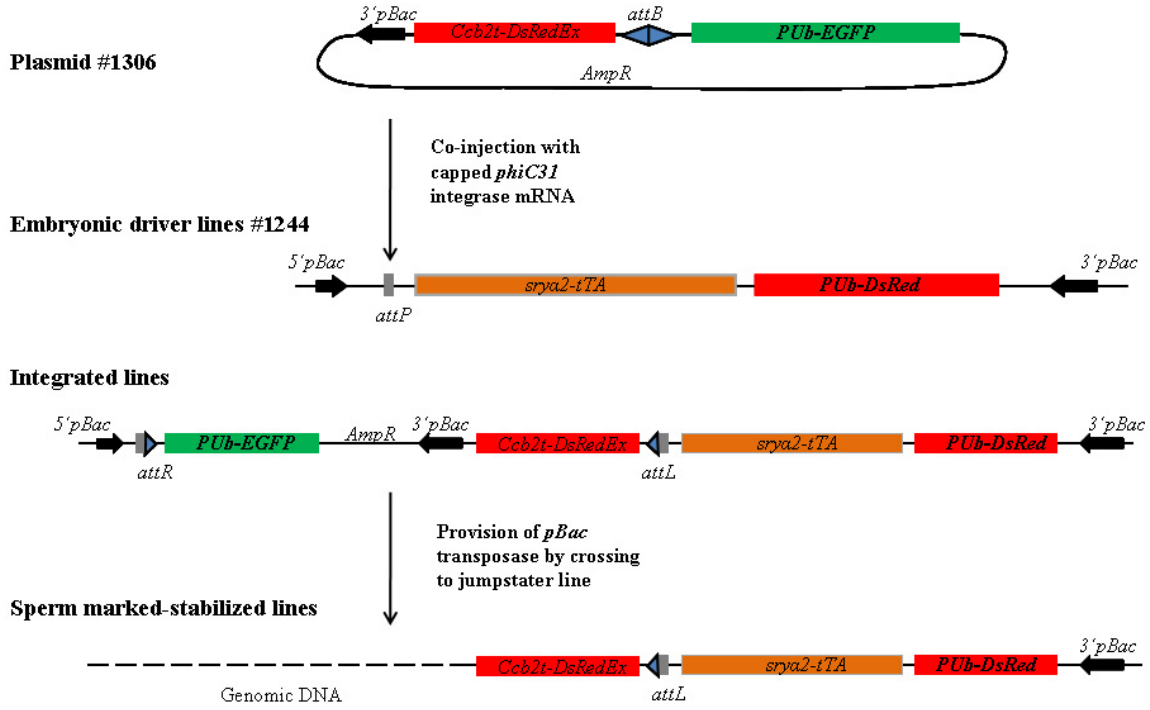

Figure 3.3.2. Strategy to add a sperm-marking cassette and stabilize evaluated embryonic driver lines, #1244_f4m1 or #1244_m2m1. Co-injection of plasmid construct #1306 (containing a sperm marking cassette) and capped *phiC31* integrase mRNA should lead to site-specific integration of the construct into the *attP* site of transgene #1244 which was randomly inserted into the genome of a wild type fly using a *pBac* transposon vector. Provision of *pBac* transposase to the integrated strain could cause remobilization of a part of the transgene (5´*pBac* and 1st 3´*pBac* ends) to leave behind a stable sperm marked driver transgene (or series of other transposition events not depicted).

3.4. Isolation and characterization of the gene *nanos* from the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae)

The strategy used to achieve site-specific integration in the medfly *C. capitata* which involves co-injection of a desired plasmid and capped *phiC31* integrase mRNA into a target line has proved to be of low efficiency (see sections 3.2 and 3.3). For the development of a more efficient site-specific recombination strategy in *C. capitata*, I isolated the upstream region of the gene *nanos*, which is a maternal-effect and germ line-associated gene known in many insects, to drive in the future the integrase in the correct tissues for germ line integration.

3.4.1. The *nanos* gene in *C. capitata*

Successful amplification of the full cDNA by degenerate primer PCR and 5' as well as 3' RACE and subsequently the genomic fragment by PCR enabled the determination of the structure of the medfly *C. capitata nanos* (*Ccnos*) gene. The cDNA of *Ccnos* is 2337bp long and consists of a 5´end untranslated region (UTR) of 162bp, an open reading frame (ORF) of 1197bp and a 3´UTR of 978bp in length (fig. 3.4.1; appendix 2.6). The gene contains four exons and three introns: (i) a 199bp exon 1 that bear both the 5´UTR and part of the ORF, exon 2 of 432bp, exon 3 of 461bp and a 1245bp long exon 4 that bears part of the ORF and the 3´UTR (ii) a very small intron 1 (62bp) and long introns 2 and 3 of 2220bp and 1189bp respectively (fig. 3.4.1).

Analysis of the amino acid sequence obtained after translation of sequences of the ORF show that the CcNOS is a 398 amino acid long protein (see appendix 2.7). Its carboxyl-terminal region contains conserved sequences and alignments of a stretch of 63 amino acids from this conserved region to NOS proteins of other cyclorrhaphan dipterans revealed high similarities: 95% for the housefly *M. domestica*, 84% for *D. virilis* and 82.5% for *D. melanogaster* (fig. 3.4.2). Also, this region contains the conserved cysteine and histidine residues which form zinc-finger binding motifs characteristic of this protein family (fig. 3.4.2). The amino-terminal region however bears little similarity to the NOS proteins of other cyclorrhaphan dipterans.

In addition, a 1.18kb long region upstream of *Ccnos* was obtained by inverse PCR on genomic DNA (fig. 3.4.3). The obtained nucleotide sequence is available in appendix 2.9. It is

expected that the promoter/enhancer elements of *Ccnos* should be contained within this amplified upstream region.

3.4.2. Expression patterns of *Ccnos* mRNA in ovaries and embryos

The detection of *Ccnos* transcripts in ovaries was achieved by WMISH of DIG-labelled antisense RNA probes to ovarioles separated from ovary bunches dissected out of unmated females. After incubation with antisense probes, there was non-detection of transcripts in the germarium (fig. 3.4.4.A1, inset) and up to oogenesis stages 8 (fig. 3.4.4A1). However, transcripts were strongly detected in nurse cell cytoplasm of the stage 10 egg chamber but not in the oocyte or its surrounding follicle cells (fig. 3.4.4.A2). Same observation was made at oogenesis stage 11 (fig. 3.4.4.A3). In stage 13, transcripts were detected both in nurse cells and gradient-wise from the anterior part in the oocyte following nurse cell dumping, but not in the follicle cells (fig. 3.4.4.A4). No mRNA detection was obtained for all oogenesis stages after incubation of ovarioles with the sense RNA probes (fig. 3.4.4.B1-4).

Expression of *nos* mRNA in medfly embryos was also detected via WMISH with DIG-labelled antisense RNA probes. During the early blastoderm stage, transcripts exhibited a gradient distribution; strongly localized at the posterior-most part of the embryo and decreasing gradually towards the anterior of the embryo (fig. 3.4.5.A). The cellularization stage revealed a more restricted localization of the expressed mRNA to the posterior pole (fig. 3.4.5.B). Transcript detection at germ band elongation was weak but localized to germ cells (fig 3.4.5.C). Incubation of embryos with sense RNA probes yielded no specific staining or detection of transcripts (fig. 3.4.5.D-F).

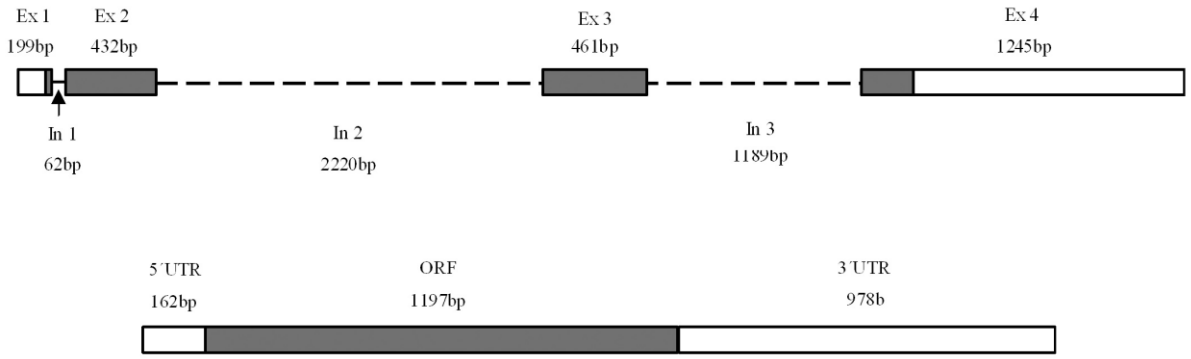

Figure 3.4.1. Structure of *Ceratitis capitata nanos* gene. Schematic drawing of *nanos* gene in *C. capitata* (*Ccnos*). The numbered letters **Ex** and **In** indicate the exons and introns respectively, with the length of each segment indicated directly below in base pairs (bp). Exons are represented as boxes and introns as lines. Dashed lines indicate introns that are too long to be drawn to scale. Below the genomic representation is the cDNA structure showing the 5´ and 3´ untranslated regions (UTR), the open reading frame (ORF) and their respective lengths. Regions of the ORF in both the genomic DNA and cDNA are shaded in grey.

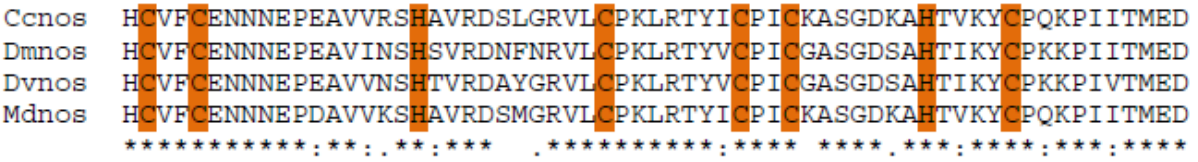

Figure 3.4.2. Alignment of the highly conserved region of CcNOS and NOS proteins of other cyclorrhapha dipterans. The conserved protein region encoded by the *nos* gene in medfly shows high similarity to those of other dipterans especially the housefly *M. domestica*. Highlighted in colour are the two CCHC zinc-finger motifs that are known to be highly conserved in many insects.

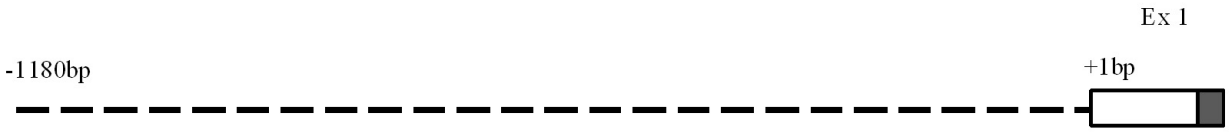

Figure 3.4.3. Schematic representation of the region upstream of *Ccnos* cDNA. The dashed line shows the 1180bp region upstream of *Ccnos* (not drawn to scale) isolated by inverse PCR. Exon 1 is represented by a box with +1 being the transcription start site. Open part of box is the 5´UTR while in grey colour is part of the ORF.

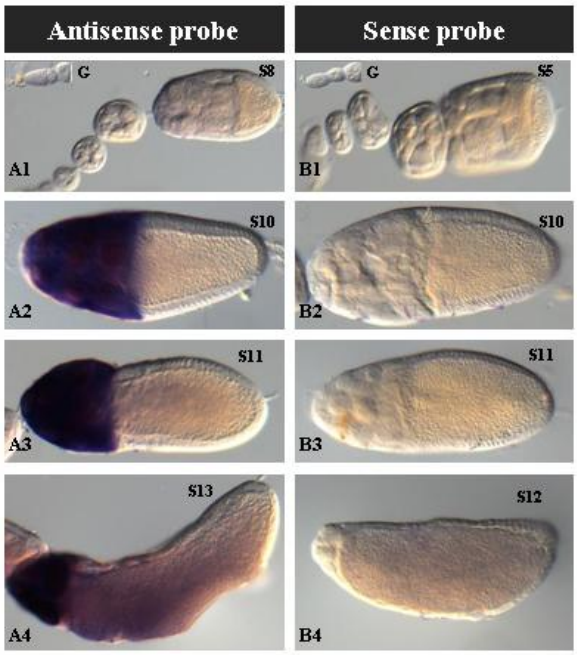

Figure 3.3.4. WMISH of *Ccnos* RNA probes to *C. capitata* ovaries. mRNA of *nos* is clearly detected during medfly oogenesis stage 10 (A2) and onwards with *nos* antisense RNA probes. Sense RNA probes gave no mRNA detection. Oogenesis stages are as described by King (1970). Inset in A1 and also in B1 is the germarium (G). Egg chambers are orientated with anterior to the left.

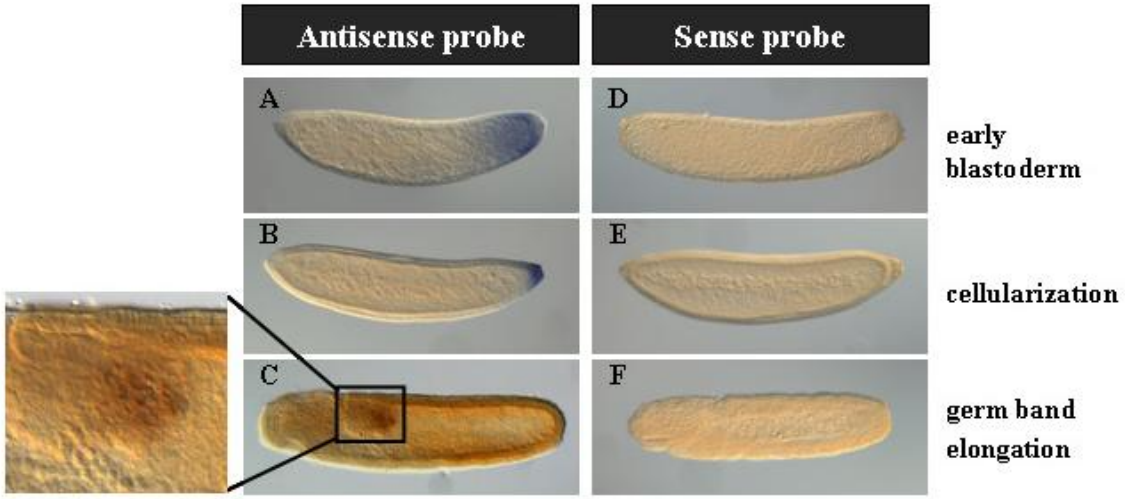

Figure 3.4.5. WMISH of *Ccnos* RNA probes to *C. capitata* embryos. *nos* mRNA has a posterior localization which is in a gradient pattern in early blastoderm embryos (A), more concentrated at the posterior pole at cellularization (B) and weakly detected around germ cells during germ band elongation (C, enlarged). Because of the weak signal obtained from the antisense probe-stained germ band elongation stage embryo, dehydration with methanol was not performed, in contrast to other stages leaving the colour of staining reddish. No transcript detection was possible in embryos incubated with sense probes. Embryos are orientated with anterior to the left and dorsal side upwards.

4. DISCUSSION

In the fight against tephritid fruit flies of agricultural importance, SIT remains an efficient, target-specific and environment-friendly method that achieves successful management of these pests. With improvements of its various technical aspects, this area-wide method of insect pest control will become much more efficient and cheaper/easier to execute. This study employed genetic tools to try to bring valuable improvements to fruit fly SIT. Its achievements and their significance are discussed below, as well as a look at the future of transgenic fruit fly SIT.

4.1. Early-acting transgenic sexing system

Fruit fly SIT has been shown to be more efficient when only males are released (Rendon *et al*., 2004), and programmes are generally faced with a major problem of efficient sex separation of adults prior to field release. Currently, the only known sexing system in large scale use is the medfly Genetic Sexing Strain GSS (Franz, 2005) that offer high benefits, though they have some fertility and fitness problems (Robinson, 2002). A transgenic sexing strain (TSS) that was developed to possibly overcome the limitations of GSSs has a shortcoming in not being able to eliminate unwanted larvae as the GSSs do (Fu *et al*., 2007). The early-acting sexing system developed in this study (section 3.1) presents a good transgenic alternative that might effectively replace the medfly classical GSSs. This system has been tested in the Mediterranean fruit fly *C. capitata* and found to function satisfactorily as demonstrated by some of the FSEL strains that showed complete elimination of females at the embryonic stage or shortly after hatching and the lethality is repressible by 10µg/ml tetracycline. Four FSEL strains #32, #33A, #33B and #43B all showed complete female removal in double homozygous conditions and can be used for medfly sex separation prior to field release. Just like GSSs, the early-acting transgenic sexing strains should provide cost effective and efficient pest management if found to perform satisfactorily in large scale tests and incorporated into area-wide SIT programmes. In addition, the males are also expected to exhibit better fertility and fitness than GSSs, while the females are also expected to be fitter than their GSSs counterparts. Besides, less manual work will be required from production

facility rearing staff as the lethality in this strain would ensure effortless removal of undesired females at the embryonic stage.

An earlier attempt to develop female-specific embryonic lethality in *C. capitata* by placing the sex-specifically spliced *Cctra-I* intron in the driver construct (see fig. 3.1.1) of a conditional embryonic lethality system (Schetelig *et al.*, 2009a) was partially functional, and remobilization of the sexing driver construct to other genomic positions improved reduction of female progeny to 30% (Schetelig *et al.*, 2011). Placing the sex-specifically spliced *Cctra-I* intron in the effector construct rather than in the driver construct, as was done in this study, led to the generation of a fully functional female-specific embryonic lethality system. The reason for this might be that the blastoderm-specific promoter in the driver construct restricts gene expression to the early developmental stage and so the transcripts produced would have only a short period to be alternatively spliced. Though splicing of endogenous *Cctra* gene is known to occur as at cellularization (Gabrieli *et al.*, 2010; Schetelig *et al.*, 2007), the auto-regulatory loop of female-specific splicing of this gene is established much later (Pane *et al.*, 2002; Salvemini *et al.*, 2009). Having the *Cctra-I* intron in the effector construct, which is not stage restricted, would ensure that enough transcripts are still available to be alternatively spliced by the time the auto-regulatory loop of female-specific splicing is established.

Sufficient transgene expression and correct splicing are also necessary to achieve a high level of female-specific embryonic lethality. Some of the FSEL strains generated using weak drivers (FSEL strain #13, for example) were not able to achieve high levels of female removal, while FSEL strains generated using sexing effector line #1402_22m1 and strong drivers (see table 1.1) showed lethality both in males and female. Mediation of lethality in both males and females suggests that the *Cctra-I* intron in sexing effector line #1402_22m1 is not correctly spliced, and remobilization of the transgene of this line to other genomic locations led to generation of new lines that mediated female-specific lethality indicating that correct splicing of *Cctra-I* in their transcripts occurred. This results show that the genomic integration site of an alternatively spliced transgene may be crucial to obtaining correct splicing. Thus, “position effects” can influence the functionality and efficiency of a female-specific embryonic lethality system by modulation of alternative splicing as well as the strength of transgene expression. The adjacent sequences immediately flanking the *Cctra-I* intron may also be important to obtain correct splicing, but this seems to be flexible; the

dinucleotides TG and GT flanking the *Cctra-I* intron (Fu *et al*., 2007) or TG and GC flanking *Cctra-I* intron in this work both achieved the expected sex-specific splicing.

There are needs for the development of sexing strains for many other insects species which to date have no sexing systems for large scale application (Robinson and Hendrichs, 2005; IDIDAS, 2004). One great hurdle expected to be crossed by the TSS is therefore the aspect of transferability to other species. Vital constituents of the early-acting sexing system exist widely among dipterans and some have even been shown to function in both insects and mammals: (i) genes that are active during cellularization abound in insects and promoters/enhancers of some of these genes for example *sryα* have been isolated and used in two distantly related dipteran species, *D. melanogaster* (Horn and Wimmer, 2003) and *C. capitata* (Schetelig *et al.,* 2009a); (ii) the heterologous *tTA* functions in *D. melanogaster* (Bello *et al.,* 1998; Horn and Wimmer, 2003), *C. capitata* (Schetelig *et al.,* 2009a; Gong *et al.,* 2005) and mammalian cells (Gossen and Bujard, 1992); (iii) The alternatively spliced *transformer* gene is a sex determination gene existing and well studied in different dipterans such as *Drosophila* spp (Sosnowski *et al.,* 1989; O'Neil and Belote, 1992), *C. capitata* (Pane *et al.,* 2002), *Bactrocera oleae* (Lagos *et al.,* 2007) and *Anastrepha* spp (Riuz *et al.,* 2007). Moreover, reports show that CcTRA functions correctly in directing female-specific development in *D. melanogaster* (Pane *et al.,* 2005), while *Cctra* transcript is recognized and correctly processed by DmTRA/DmTRA2 (Fu *et al*., 2007). Going by these reports, one can envisage that such a system as described in this study would be relatively easy to develop for other tephritid pest species. A direct transfer of the original *C. capitata* female-specific embryonic lethality system to another tephritid species may be possible. In such attempt however, a possible case of non-functionality of promoters/enhancers, as earlier seen with *D. melanogaster sryα* promoter's non-functionality in *C. capitata* (Schetelig *et al.,* 2008b), might be encountered and the use of endogenous constituents would then be the best option. Current effort to generate a female-specific embryonic lethality system in *Anastrepha* using endogenous constituents has yielded success (Schetelig and Handler, personal communication).

Early-acting transgenic sexing strains (TSSs) can also be used for "Release of Insects carrying a Dominant Lethal" (RIDL) or genetic sterilization (Thomas *et al*., 2000; Fu *et al*., 2007) in addition to being applied for the purpose of sex separation. The FSEL strains generated in this study, such #43B that showed complete removal of females in both double heterozygous and

double homozygous conditions, also fit well into this scheme since all female progeny of released double homozygous transgenic males following their mating to wild type females are expected to die. However, the subsequent generation of male-only transgenic individuals (F1) would be double heterozygous and though they will be present to continue control of target pest population without new releases, their female progeny following mating to wild type females (F2) would partially survive. Therefore, a better choice would be to use the early-acting TSSs for sex-separation in mass-rearing facilities coupled with sterilization by ionizing radiation. Doses of radiation applied will then have to be calculated as to reduce the adverse effects associated with this method without compromising sterility. An ideal transgenic system that should achieve efficient "sterilization" and may be combined with this early-acting sexing system is the semen-based lethality system (SEMELE) proposed for mosquito control by Marshall *et al*. (2011), but semen-based lethality systems are still theoretical and yet to be developed in any insect.

4.2. Site-specific recombination

Because SIT involves field releases of males and transposases exist in nature, it is important that transgenic insects for release meet the requirements of transgene stability to preserve their engineered traits and also address ecological concerns. With respect to these, the evaluated and most potent sexing effector line of the early-acting sexing system was stabilized through the ability to integrate genetic materials site-specifically into an *attP* site using the *phiC31* recombination system (Thorpe *et al*., 2000) and post-integrational deletion of *pBac* transposon ITRs (Handler *et al*., 2004) (section 3.2). Though the original sexing effector line #1402_22m1B was found to carry two copies of the same transgene randomly inserted by *pBac* transposition, it was possible to site-specifically integrate a construct bearing an additional 3´*pBac* end into the *attP* site of one of the transgene insertions and subsequently stabilize this transgene and also remove the second unmodified transgene. The stabilized line #1402_22m1B_stab_f4 showed good transgene stability on exposure to *pBac* transposase and would be better suited for SIT programmes compared to the potentially unstable original strain as it addresses ecological concerns and also satisfies the requirement for transgene stability.

With regards to female-specific embryonic lethality, the stabilized line also performed satisfactorily despite initial concern over removal of one of the transgene copies; considering "position effects" on transgene expression (Heinrich and Scott, 2000; Schetelig *et al*., 2011), I was not certain which of the trangene insertions in two different genomic positions had a better killing power. Fortunately, the single stable transgene remaining after post-integrational stabilization and removal of the second transgene was potent enough to cause elimination of all double homozygous female progeny when crossed to an embryonic driver line. However, the embryonic driver line used with this stabilized sexing effector line in the lethality test is still potentially unstable and would have to be made stable for the strain to be practically usable in operational SIT programmes. Stabilization of efficient embryonic driver lines following the same strategy used for the sexing effector presents an opportunity to add to it a new gene-of-interest such as a sperm-marking cassette (Scolari *et al*., 2008) (see section 1.3, 3.3). This will enable the creation of an optimized medfly strain highly desirable for operational SIT.

The current strategy for achieving site-specific integration in medfly using the *phiC31* recombination system involves co-injection of both the desired plasmid and capped *phiC31* integrase mRNA (Bischof *et al*., 2007; Schetelig *et al*., 2009b). This strategy is however not very efficient and another strategy developed in *D. melanogaster* using regulatory elements of maternal-effect genes *vasa* (*vas*) and *nanos* (*nos*) has been shown to be of higher efficiency and convenience (Bischof *et al*., 2007). Regulatory elements of *nos* have also been utilized to control tissue and sex-specific expression of a transposase in the germ line of the yellow fever mosquito *Aedes aegypti* (Adelman *et al*., 2007). To upgrade the site-specific integration strategy in medfly to a high level of efficiency and convenience, regulatory elements of medfly maternal-effect genes are needed. In this study, the medfly *C. capitata* ortholog of the gene *nos* has been successfully isolated and characterized and a 1.18kb fragment upstream of the gene, which should contain the regulatory elements, was also obtained. The *Ccnos* transcripts are maternally-derived and associated with the germ line in embryos, showing that this gene is similar to the *nos* genes in other insects (Curtis *et al*., 1995; Calvo *et al*., 2005). The obtained *Ccnos* upstream region will be invaluable in the construction of an efficient *phiC31* site-specific integration system in medfly by allowing maternal and "endogenous" provision of *phiC31* integrase spatially around embryonic germ cells. Such a system should achieve faster and better site-specific transgene integration and permit easier combination of different transgenic traits in a single strain.

4.3. Future of transgenic fruit fly SIT

Over the last decade, tremendous progress has been made in the engineering of insect strains expressing various traits. Molecular biotechnology has been able to offer different transgenic strains possessing valuable qualities that are difficult to be realized by other means. Contrary to concerns that existed over their performance compared to wild type strains, some of such transgenic strains for example two different strains developed for medfly have been shown to perform comparably or even better than their wild type counterparts when tested in field cages (Schetelig *et al*., 2009a; Morrison *et al*., 2009). Therefore, utilization of such strains in SIT programmes should readily achieve reasonably good output. But the actual use of transgenic insects in large scale SIT pest control programmes has been somewhat slow, though SIT using transgenic insects with negative selectors such as lethality is considered environmentally safe since the released insects should not persist in the environment nor their genes inherited by subsequent generations. Public and expert concerns and caution do exist over the use and field release of genetically modified insects for pest management (Knols *et al*., 2006; Enserink, 2010), and sometimes such concerns may put serious delays or stall the application of outcomes of years of serious scientific research. A known example of the extent to which concerns, if they get overblown in the media or turn political, may affect control programmes is the abandonment of a World Health Organization-funded mosquito genetic control program shortly before commencement of releases due to claims of biological warfare (WHO, 1976). The large-scale field release of about 3.3 million sterile transgenic *Aedes aegypti* mosquitoes by Oxitec Ltd in Grand Cayman to control dengue fever has also generated so much controversy, though many also agree that it was a success (Enserink, 2010; Subbaraman, 2011; Benedict *et al*., 2011). Subsequent to the Grand Cayman, GM mosquito field releases have also been made in Malaysia and Brazil (Reeves *et al.*, 2012). Many countries are reportedly evaluating the prospect of carrying out field releases of GM insects, prompting calls for more transparency in GM insect release activities and better scientific regulatory standards to instil higher public confidence in GM technology (Reeves *et al.*, 2012; Lehane and Aksoy, 2012). While most experts agree on proper regulation, some also argue for sound rational and practical consideration in assessments and regulation of GM insects (Alphey and Beech, 2012).

In the light of the above recent transgenic mosquito releases, it seems that the future of transgenic fruit fly SIT looks bright. Strong hope of actual field use of transgenic fruit flies exists following a record of decision in the United States of America that authorizes the development and use of genetically engineered fruit flies and pink bollworms in SIT application for plant pest control (USDA-APHIS, 2009). So far, field releases have been conducted for the pink bollworm *Pectinophora gossypiella* in Arizona, USA and the trials show that the transgenic strain performed well under field conditions (Simmons *et al*., 2011). To my knowledge, no large scale field release of any transgenic tephritid fruit fly species has yet been made, but it will only be a matter of time.

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6. APPENDICES

Appendix 1: Tables

1A. Female-specific lethality in double heterozygous progeny

Sexing effector	Driver	FSEL strain	Tet.	Replicate 1 ♂:♀	Replicate 2 ♂:♀	Replicate 3 ♂:♀
1402_21m1	*sryα2-tTA*_f2m1	#21	No	240:212	178:128	310:285
1402_21m1	*sryα2-tTA*_f4m1	#31	No	165:95	154:73	263:138
1402_21m1	*sryα2-tTA*_m2m1	#41	No	82:80	242:235	161:134
1402_21m1	*CG2186-tTA*	#51	No	179:193	156:139	249:215
1402_21m2	*sryα2-tTA*_f2m1	#22	No	235:194	233:157	240:241
1402_21m2	*sryα2-tTA*_f4m1	#32	No	251:72	261:60	333:105
1402_21m2	*sryα2-tTA*_m2m1	#42	No	31:42	69:69	56:64
1402_21m2	*CG2186-tTA*	#52	No	69:50	63:47	309:152
1402_22m1	*sl1-tTA*	#13	No	87:109	124:132	284:214
1402_22m1	*sryα2-tTA*_f2m1	#23	No	198:189	184:190	218:187
1402_22m1	*sryα2-tTA*_f4m1	#33	No	0:0	0:0	0:0
1402_22m1	*sryα2-tTA*_m2m1	#43	No	0:0	2:0	1:0
1402_22m1	*CG2186-tTA*	#53	No	0:0	75:61	71:70
1402_22m1A	*sryα2-tTA*_f4m1	**#33A**	No	150:1	573:0	451:0
1402_22m1A	*sryα2-tTA*_m2m1	#43A	No	683:50	416:29	532:2
1402_22m1B	*sryα2-tTA*_f4m1	**#33B**	No	479:5	254:0	307:0
1402_22m1B	*sryα2-tTA*_m2m1	**#43B**	No	622:0	375:0	586:0
1402_22m1C	*sryα2-tTA*_f4m1	#33C	No	250:182	360:322	299:287

1B. Female-specific lethality in double homozygous progeny

Sexing effector	Driver	FSEL strain	Tet.	Replicate 1 ♂:♀	Replicate 2 ♂:♀	Replicate 3 ♂:♀
1402_21m1	*sryα2-tTA*_f2m1	#21	No	257:66	709:490	260:129
1402_21m1	*sryα2-tTA*_f4m1	#31	No	344:0	337:19	1106:76
1402_21m1	*sryα2-tTA*_m2m1	#41	No	320:36	396:28	365:34
1402_21m1	*CG2186-tTA*	#51	No	259;3	335;135	640;245
1402_21m2	*sryα2-tTA*_f2m1	#22	No	817:505	336:79	319:72
1402_21m2	*sryα2-tTA*_f4m1	**#32**	No	476:0	821:0	435:0
1402_21m2	*sryα2-tTA*_m2m1	#42	No	306:1	120:1	202:4
1402_21m2	*CG2186-tTA*	#52	No	868:193	368:23	160:28
1402_22m1	*sl1-tTA*	#13	No	395:207	102:74	156:109
1402_22m1	*sryα2-tTA*_f2m1	#23	No	254:211	88:85	297:252
1402_22m1	*sryα2-tTA*_f4m1	#33	No	0:0	0:0	0:0
1402_22m1	*sryα2-tTA*_m2m1	#43	No	0:0	0:0	0:0
1402_22m1	*CG2186-tTA*	#53	No	0:0	0:0	0:0
1402_22m1A	*sryα2-tTA*_f4m1	**#33A**	No	619:0	373:0	394:0
1402_22m1A	*sryα2-tTA*_m2m1	#43A	No	*	*	*
1402_22m1B	*sryα2-tTA*_f4m1	**#33B**	No	524:0	406:0	401:0
1402_22m1B	*sryα2-tTA*_m2m1	**#43B**	No Yes	3481:0 318;341	2122:0 214;220	2712:0 297;303
1402_22m1C	*sryα2-tTA*_f4m1	#33C	No	224:133	240:180	473:159

***** No double homozygous individual for this combination was got. In bold are lethal combinations that achieved complete female elimination.

Appendix 2: Sequences

2.1. Genomic DNA sequences flanking *pBac* insertions in sexing effector #1402 lines

Restriction site for enzyme *MspI* used to perform digest on genomic DNA is underlined, while *pBac* insertion site **TTAA** is in bold. Sequences are in 5´ to 3´ orientation.

#1402_21m2

CCGGTACGTTAATTGCTGTTTCGTTAAGCTAAAAGCAGACACATATAACGCACTCGTCTGACTGGTGTCGGCAGA
TGCTGTTCCGCATTCGTGGACGCTTCATGGGAGGGCATGGATAAGAGTAAAGGGGGGTGGGTACTTAACAAGTGC
ATCTGCGTTTTGTTTTCCGTCAACTTTCGTATACATACACACTTAGATGTGTATAGACATGTACTCGTACATGTC
TTTGCGTATGTGCGGTGGTATGTGCGCATTACTCTTCTTTGCTTTTGAAAATAAGTAGTGAATTTTTGTTATGGC
TGCGGTTAACATAATTGAGCACTCCCACAAAATTATAGTCAAATACAATAAAAAAAATTATTGTTATCACTTTTT
ATATATGTATGTATGTATGTATTTGGGTAGCTTAGTTCTCTTATGCAAATACATTTGTATTATATTCAGAAATAT
ACATATACATACATACATACGTGTATTTGTGTGTTGGTCATATTTATCATTTTTTACATACGTACATATCCACAT
AAAACTGATATGAATACATTATTTTATTGAAAATATCTAATTGTTTTCAAGCACTTAATGGATTTTGGAATAAAA
TTTTACATTCAAACACTTGTATAAATAGATCCAGAATCAGAGAGCAAAAATTCTCTTGAACAAAGCTAATTTCAT
AAGTCTAAAATTAATTTATCGTTGTGTTGCTCTGTGATCAGCGCAATTTCATACGAAAATTAATCAGCTTTTGTT
AAGCAGAGCAACAATTGTGAAAATAGAAAGCGTATTGGTAAAACTATAATGAAGTACTCGTTTTAAATTTTTCTT
GAACCCATAACCCAAACTCCAAGAGCCTTATCCATAGGCTCGTGCCCCATTTCGCCCATCTTTCACGACTTTGCA
TTAACATTTTTTTTTTTGATGAAAATTGTAGTATTATATGATGATGATGAGTGTTCAAGGCAAGTAGTACATACT
GTTATATAGGTACGGGGAATTAATCAGGGGGAAAGCCTACTTTAAGGCGTTAATGTAACGCGACTGTCTTCTGCT
GAAGTTTTTTTTTTGGAGGGAGGGCGGCAGAAAAGCATCGACAACGTATGAGGACCATGCACTTCTCAGTGAAGA
TTGCCTGGCAAACTGGAGAAATAGAATCAATGAATTAGATTGTAACATTTACGTTACCCTCACTAACATGCCCTA
CGATTAACAGGCCTATTCTGTTGAGGTAAAAAAAATTTACTTTGGTATTAAAATTTACCATTCAGTAAATTTCGT
TACCGTTTCGTTACCATTTCGTATTCTGAGCTCAGTGTCACCCTGTAAATTTTGTTACCAGCGAAACAGGAAGTA
ACAAAAATATCAGCTGTTTGATTTTGTGTTGTAATTCTGAGAAAAAATAAAAAACCAAAATGGGTAGGCATAGGC
ATATTTTAGGCATGGAATTCATTGAAGATGAAGAAAAAAGTACAAAGAGTTGTATTTTAAGAAGTTTTCGAGACT
AAAAAAAGCCCTTTAATTTGTAAGATAACGTCTTCATTCAGCATTTTTTGTTATCCAGGCATATTTTCCATAATT
AGGTTTAAATATTTATGATTTAATAAACACATATATAAAACTTATTTATTTTGAACTTATTTATTTTTTCGTTGT
GAATATTTTTCTTTTTACTTTATCCGCTGGCAAATGTAATTTGTCATCGAAGAAAAAAGCAGTGCTGCCAAATTT
TTTTCAAACATTGTAACCAGTTCGTCAGAATACCAAATTCAATTTCGGGTG**TTAA**..***piggyBac***..**TTAA**AGAA
AAGTAACAAAATTTACTTTACCGG

#1402_22m1A

CCGGATCAGGATGATTTTAAAATAATAAATGTGAAATAGATAACTTGCTAAATAATAATAACCAACAAATAATAA
TTAATAGATCTATATTAGAGAGAATAAATAACTTAACACATATTACAAATATAGTCTTAAGCGAAGTAAAATCGA
GTGATGCTATAAAGTTAGAATTAGAAAGAAATAGTGAGAGAAAATTAAATTATATTAAGGAAGAGTTAAAAAATG
TAGACTATTCAATATAATGGGCAAAAGCCAAAACTGTAAACTCATTTATACTATCGAATGAAGAAATCAATGTTA
TTAAGGAAAAATTGGAAAAACAACAAATGCCTTACATAAATACAATAGAAGCTATAGAATTTAGTGATGCAAAAA
TCGCATCAAATTTATCATCCATAATTTACATTGTAAAAGTTCCTTTTACAATCAAAGAGAATTGTAAATCAATAA
TAAAAAGGCCTGTAAAACAAAGTAATATAGTTATTAAACTAGAATCTAATTGTTTGTCACAAAAAAAAATTTAAT

ATAAAGAATAGTTGTAAAAACCTAAATGATGTATCTTTATGTAAACAAGAAAACATAGAAAAAGTCACACAAAGC
AGCTGCATACCAAAATTACTTAGGGAATATTCAGCAAACTGCACAACCATCAATGGGGCGTCATATACCAACAAT
AGAAGAGATAAATCATGGCCTTATACTTTA**TTAA**..***piggyBac***..**TTAA**GGAGTTCTGCAAAGATCGAAATAAA
TGGTAGTCGGATGGTGCAAGGTCAGGGCTATATGGTGGATGCATCAAAAGTTCCCAGCCAAGCTCACTCAGTTTT
TGGCGAGTGACCAAAGAAGTGTGCGGTCTAGCGTTGTCCTGGTGGAATATGGCACCTTTACGATTGACCAATTCT
GGTCGCTTCTCCTTGATGGCTGTATTCAATTTGTCCAACTGTTGACAGTAAACATCCGAAATAATCGTTTGGTTC
CTTGGAAGCAGCTCAAAATATACGACACCCTTCCAATCCCACCAAACAGACAGCATAACCTTCTTTTGGTGGATA
TCAGCCTTTGAAGTGGTTTGAGCTGGTTCACCATCCTTGGACCATCATCGTTTTCGACTAACGTTGTTGTAAACA
AACCATTTTTCATCTCCAGTTATGGTTCGTTTTAAAAACGGATCGAATTCATTGCATATCACAAGTGTTGATTCG
GTGTGTTAAATGAATTTCTTTCAATACATGTGGTACCCAAATATCAAGCTTTTTCACCAGTCCAAGACCAATATT
TAACTTCTCTCCTATCTCACGCTCAGCTACATGACGAACCAATTCGATTAATGCTTTGATTTGGTCATCATCAAC
TTCATTTGGCCGACCTGAACGGGGCTCCTCCTCCTCTCCTCTTTATGTGAAAAATCTCCAGAACGGAATTTGCGA
AACGAATTTTGACACTGTCTTCCTTTAAGGCTTCATCACCATACACATCTCGTAACTTTTTAGCCACCTGCTCCG
G

#1402_22m1B

Transgene 1

CCGGAACGGTCAATTTCCCCCTTAGTGGGTAAGGATGGTTTCCAGGTTTCGATGGATGTGGCGCATTTTAAACCA
AGCGAATTGACTGTCAAGGTAGTAGATAATAGCATAGTCGTGGAGGGGAAGCATGAGGAGCGTGAGGATGACCAC
GGTTACATTTCTCGTCATTTCCTTCGACGTTACACGCTTCCTAAGGGATATGATGCCAACAAGGTAATGTCGTCG
CTTTCATCAGATGGAGTATTGACTGTTATGGCACCTAAGCCGCAGTTAGAAGACAAGTCCAACGAACGTCATATT
CAAATACAACAAACTGGTCCAGCACACTTGAATGTTAAGGACAATACCGAAGAGAAGAAAAAGTAATCTCATTAT
ACGTCGTGCAAAGATCATTCATAAAACATCGTACATGCATCATCTCGAGCTGGCCTGGAGTTTAGTTTAATACTT
ATTTTGCTATCAATACGCTTAATTTTCTTAAT**TTAA**..***piggyBac***..**TTAA**GACATAGTTTGTATTGAAAAGAA
GTAACACTCGAATAAAAAAAATTATTTTTTCTTAATAAAAAGCTTCAGTTATTTTATGGTAAGACGCCTGGGTTA
CATGGAATCCGTTGTGTTGGATTATAGGTATGTACATAAATACATACGAGTATAGTTAAAATTTACGTAAAGGAA
ATATTAGAAAAAAATCAAGCTCAAAAACTCACCAAATACCAACAAACATTTTTGGTCACCTTTCGAATAGAAAAC
ACTACTATTATGTTCTTGACTCCTTTCCGAGCTTTCAAAGCGCGCTAGATGGTCAACGTCCAAATTATAGAAAAT
TAAATAAGAAAGGGAATGGCGATCCCTCATAAAAATTCAACAACCGATCGAGGGTGACTCAGCAAAAACTGTAGG
TTCCTCAAGTTGTTAATATCAAGACAAGGCTCACCACAAATTAGTTGAGTTCGACCGAAAATCGCTTCAAAGGTT
CTACAGCGCCAATTATATATGTATACATGTATCTACGTATTTGTTATAAACTATCCACGAGGCGGCTCGCCATAT
TTTTATGAAATTTTGAAAACATACAAATATACAAAAATCTGTATGTATTCATATGGTCCGTTTTCTTATTGCCGG

Transgene 2

CCGGCTCATGGGCCAGTTATAGCTGTGATAGTATGGCGAAATGGCCGTAGTCAAACGGTCCATATCGTTAATCAA
ACTCAACATTGCTGGAATTGTAGCCATTGTTTTTTCCTGAACTGAAAAATTAATTTACTTTTTTTGTATGACTTC
ACTTAATTAGGCAAGTTTTAACTCCCGCAGAAATACTGT**TTAA**..***piggyBac***..**TTAA**TTTAAATCGCTTCAAT
GTTTGTATGCAACTGTTGGGTACGTTCTCGTTATGCCGG

2.2. DNA sequences of *attP* site amplified from sexing effector lines using primers mfs373/360

In bold is *attP* sequence while other sequence (not in bold) belongs to other part of plasmid #1402. Sequence is in 5´ to 3´ orientation.

AAGTCCACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCGGCCGGCCTTGGCGC
GCCAAGCTTAAGGTGCACGGCCCACGTGGCCACTAGTACTTCTCGAGCTCTGTACATGTCCG
CGGACTAGG**GTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGTC**GC
TAGGCC

2.3. DNA sequences of attachment junctions (*attR* and *attL*) amplified from integrated lines #1402_22m1B_int using primers mfs373/372 and mfs330/360 respectively

DNA sequences that previously belonged to *attP* site are in bold while those from *attB* are underlined. The rest of the sequences are part of constructs #1402 (for *attR*) or #1252 (for *attL*). Sequences are in 5´ to 3´ orientation.

attR PCR fragment

ATCTTGACCTTGCCACAGAGGACTATTAGAGGTAAGAATAAACATTGTTGGTCAACTTCAAAGTCCACGAGGCGT
AGCCGAGTCTCTGCACTGAACATTGTCAGATCGGCCGGCCTTGGCGCGCCAAGCTTAAGGTGCACGGCCCACGTG
GCCACTAGTACTTCTCGAGCTCTGTACATGTCCGCGGACTAGG**GTGCCCCAACTGGGGTAACCTTTG**GGCTCCCC
GGGCGCGTACTCCACCTCACAGATCTCGAGCTCAA

attL PCR fragment

GCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAAAGACTTGAGCAGAGACACGTT
AATCAACTGTTCAAATTGATCCATATTAACGATGGGCCCTATATATGGATCCTGCGGGTGCCAGGGCGTGCCCTT
G**AGTTCTCTCAGTTGGGGGCGTAGGGTC**GCTAGGC

2.4. DNA sequence of a fragment amplified from the integrated line #1402_22m1B_int using primers co39/mfs372

Underlined are amplification primers, in bold are sequences of 5'*pBac* end and in italics are sequences of *attR* junction. Sequence is in 5´ to 3´ orientation.

GGAGTATTGACTGTTATGGCACCTAAGCCGCAGTTAGAAGACAAGTCCAACGAACGTCATATTCAAATACAACAA
ACTGGTCCAGCACACTTGAATGTTAAGGACAATACCGAAGAGAAGAAAAAGTAATCTCATTATACGTCGTGCAAA
GATCATTCATAAAACATCGTACATGCATCATCTCGAGCTGGCCTGGAGTTTAGTTTAATACTTATTTTGCTATCA
ATACGCTTAATTTTCTTAAT**TTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTG**
CTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCGCTTGGAGCTCCCGTGA
GGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGCATGATT
ATCTTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCATGTTCTACTTACGTGATAAC
TTATTATATATATATTTTCTTGTTATAGATATCGTGACTAATATATAATAAAATGGGTAGTTCTTTAGACGATGA
GCATATCCTCTCTGCTCTTCTGCAAAGCGATGACGAGCTTGTTGGTGAGGATTCTGACAGTGAAATATCAGATCA
CGTAAGTGAAGATGACGTCCAGAGCGATACAGAAGAAGCGTTTATAGATGAGGTACATGAAGTGCAGCCAACGTC
AAGCGGTAGTGAAATATTAGACGAACAAAATGTTATTGAACAACCAGGTTCTTCATTGGCTTCTAACAGAATCTT
GACCTTGCCACAGAGGACTATTAGAGGTAAGAATAAACATTGTTGGTCAACTTCAAAGTCCACGAGGCGTAGCCG
AGTCTCTGCACTGAACATTGTCAGATCGGCCGGCCTTGGCGCGCCAAGCTTAAGGTGCACGGCCCACGTGGCCAC
TAGTACTTCTCGAGCTCTGTACATGTCCGCGGACTAGG*GTGCCCCAACTGGGGTAACCTTTGGGCTCCCCGGGCG*
*CGTACTCCACCTCACAGATCT*CGAGCTCAA

2.5. DNA sequence of fragment amplified from the stabilized line #1402_22m1B_stab using primers co39/co33

Underlined are amplification primers, in bold is the **TTAA** insertion site of *pBac* remaining after correct excision of 5´*pBac* and first 3´*pBac* end (see Figs. 1 & 2B) and in italics are sequences of *attL* junction. Sequence is in 5´ to 3´ orientation.

GGAGTATTGACTGTTATGGCACCTAAGCCGCAGTTAGAAGACAAGTCCAACGAACGTCATATTCAAATACAACAA
ACTGGTCCAGCACACTTGAATGTTAAGGACAATACCGAAGAGAAGAAAAAGTAATCTCATTATACGTCGTGCAAA
GATCATTCATAAAACATCGTACATGCATCATCTCGAGCTGGCCTGGAGTTTAGTTTAATACTTATTTTGCTATCA
ATACGCTTAATTTTCTTAAT**TTAA**ATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCTGTCGTGAATACCGTA
TATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAGTTTTTCGCTCATCGACTTGATATTGTCCGA
CACATTTTCGTCGATTTGCGTTTTGATCAAAGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCAT
ATTAACGATGGGCCCTATATAT*GGATCCTGCGGGTGCCAGGGCGTGCCCTTGAGTTCTCTCAGTTGGGGGCGTAG
GGTC*

2.6. Sequences of *Ccnos* full length cDNA

5’ and 3’ UTRs are in italics, while ORF is in bold. Sequence is in 5´ to 3´ orientation.

ATTTGTTAAATTCTTTCAGTAAGTTGGAAGCAATATATTTTGCTGCACTCAAGTGTTTTAAATCGTACCTGTTCG
GTGAAATCTATGAAGATAGATATTTTCATCGAGTGTATTGAAAGTACAACGTAACGGATTGTAATACTGATAGCT
*GTAGTAATAATT***ATGCTTGGAGCCAAGAGATATGCTACTTCCCGAGGGGATGCCGTTTCTTTTCTTGAATTTGGA**
TCTCTTGATATTAAATGCATACCGGAAATAGGTGTGGATAGAACAATTGCAAGTAACTGCTCACCTAGTCTTTCG
CCTACATCTACGGAATCGTCAATATCGTATCTTTCGTCAGATATATTGAGTTCATATTCGCCCACTCCTATCGGC
AGATCTATCACTCCCACTTGGAAAACCATAAATAATTTAAGTAGCGATGGAGTGCTGGACATGAACGTGCCTGTC
TTAATGAAACGCAACGAAAATGTTCATCAGGGACATAATGAGGGATCTAATGGACAATTAGCTCTGCAACAGCAT
TTTGGGAAATTATTATACTATACACAAAACCAAATGGAACAGAAAGACGAAATATCCAAGTCCCTTAAAATGTTC
GCTTTGTTGGCTTCTTGCAAATATGAAAACGATCCGTTAGAAGCAACTAAAGACTCTCCAGTAGATGACATAATG
GAAGAATTTTATTGTAACGGATATGTTGCTGACGAAAAAAATTTATTTCAACAAGGAAAAGCATGGCATAATCCT
TACTATAACTTCAATACAAATATGTGCAACAATAATGGCAACCAGAGCAATTTAAGTTTTGGAGTTATAAATTAT
GGACATAACAATAGTAGTGTCGCTGCTAATCATAGTATTAACAACAACAATGCTTTGAATCAACATTGTTTTGAA
GCCAAGTTAGGTATGCTGAACTTACCGGCCCCGTCGCCTTATTTCAATAGCAACATGGCATTGCAGATCCCACAG
CAAAATACTGCTGGAATCACAGTTGCCGCAGCAGCTGCTGCCGCCAATATTGACTACAACAACAAAAACTCAAAA
AAGGCACAGAAGCGGTATAGCAACGCAAAGTTGGATAAGTTCTCTGCAGTAAAACACTGTGTTTTTTGCGAAAAT
AACAACGAACCGGAAGCTGTAGTGAGAAGTCATGCCGTACGCGATTCGCTAGGACGTGTTCTATGCCCCAAATTA
CGGACTTACATTTGCCCGATTTGTAAAGCCTCGGGGGATAAGGCGCATACAGTTAAATATTGCCCGCAAAAGCCT
ATTATTACAATGGAAGACGCAGTAAAAGCTGAATCTTTACGCCTAGCAAAGAACTTGTACTTCAAACAAGGAATG
AAAGTATAA*GAAATTAGTTTTTTAAATTCTATCTTAATAGAAATTTCTTTAAAAAGAAAATTTTTCGTTTTTGTA*
AATATAGTTTCTAAGAGTCGTACAGAAAAATGAATGTACGTTAAATTATTAATTATTATTTACTTTTCGTTTTAG
TAAATAAATTTTGTTAAGGGTAAAAAAATGTATATGAACGCACATATATGCCTTAAAAAATGAATCGTTTAATTT
AATAAACCATTCACGTATTTAAATTTCCTGCTTAATATAGATAAGTATAAATTGAAGTGAAAGCAATATTTTTCC
TTATTAAATAGCGCAGAGTTGGTTAAACTCATTTAATTTTCTGATGTGTTGAAGTGATAATTGACACTTAAATTA
AAATATTGGCATACTTCAATTGATCTCATTGAACTGTTCAATATAACATTATACTTATAAATTACATATCTTTAC
TATATTAATAACTTAACAGCTCGTAATTCATTAGCTTTGCAGCTACTCGATTTTAATATTTTAATGTATTGTTCT
TTATTGAAGTATTAACCTGCTGGTGGCGTTAAAATCGATTTAGTGGCTCATGCCGCTTTTTATATATGAACTTAT
ATATTGAACTTGTGCCTAAAAAATAAAATTTTTTAATACGCTCATTACAAAACTATCGCATACAATGACAAAATA
TATTCTTTTTACGAACATATAATGAATCTTTCGCTATTCATAATTGCTTATATTTTGTATTGAGTTTAGTTTCTA
GAATATCATCGCCAAGATACTCTAAAATGCTGGTATTTTTTGTGTAATTTACTTAAGGTGGTATATATATGTAAA
CGTATTTGGAGTTGAATTGAAATGGTAAATTATGTAATTACCATAAATATAAGTTTTGTTTTACATACATACGTA
CCATGTAACTATCTACAGCTATTGTAAATAAATAAAGAGTATTCAAACTTATCTTTCTAAAAAAAAAAAAAAAAA
AAAAAAAAAAGT

2.7. Amino acid sequence of *Ccnos* gene

MLGAKRYATSRGDAVSFLEFGSLDIKCIPEIGVDRTIASNCSPSLSPTSTESSISYLSSDIL
SSYSPTPIGRSITPTWKTINNLSSDGVLDMNVPVLMKRNENVHQGHNEGSNGQLALQQHFGK
LLYYTQNQMEQKDEISKSLKMFALLASCKYENDPLEATKDSPVDDIMEEFYCNGYVADEKNL
FQQGKAWHNPYYNFNTNMCNNNGNQSNLSFGVINYGHNNSSVAANHSINNNNALNQHCFEAK
LGMLNLPAPSPYFNSNMALQIPQQNTAGITVAAAAAAANIDYNNKNSKKAQKRYSNAKLDKF
SAVKHCVFCENNNEPEAVVRSHAVRDSLGRVLCPKLRTYICPICKASGDKAHTVKYCPQKPI
ITMEDAVKAESLRLAKNLYFKQGMKV

2.8. Sequence of the genomic region of *Ccnos* gene

5´ and 3´ UTRs are in italics, exons are in bold letters while introns are not in bold. Sequence is in 5´ to 3´ orientation.

ATTTGTTAAATTCTTTCAGTAAGTTGGAAGCAATATATTTTGCTGCACTCAAGTGTTTTAAATCGTACCTGTTCG
GTGAAATCTATGAAGATAGATATTTTCATCGAGTGTATTGAAAGTACAACGTAACGGATTGTAATACTGATAGCT
*GTAGTAATAATT***ATGCTTGGAGCCAAGAGATATGCTACTTCCCGAGGGG**TATGTAGAATCGGATTGCCCGTTATT
TGTAAGAGTTTATTTTGGTCATGTCTCTCTTAAAGG**ATGCCGTTTCTTTTCTTGAATTTGGATCTCTTGATATTA**
AATGCATACCGGAAATAGGTGTGGATAGAACAATTGCAAGTAACTGCTCACCTAGTCTTTCGCCTACATCTACGG
AATCGTCAATATCGTATCTTTCGTCAGATATATTGAGTTCATATTCGCCCACTCCTATCGGCAGATCTATCACTC
CCACTTGGAAAACCATAAATAATTTAAGTAGCGATGGAGTGCTGGACATGAACGTGCCTGTCTTAATGAAACGCA
ACGAAAATGTTCATCAGGGACATAATGAGGGATCTAATGGACAATTAGCTCTGCAACAGCATTTTGGGAAATTAT
TATACTATACACAAAACCAAATGGAACAGAAAGACGAAATATCCAAGTCCCTTAAAATGTTCGCTTTGTTGGCTT
CTTGCAAATATGAAAACGGTAATTAGAAATACATATGTATGTGTGTTAACGTTAATCTACTACGTATCTGATTTT
TCTAACTGCGATAGTATTTGTTTTTAGCCTGTCACCGTTAAAGGCGACTGTCTGTCTGACTTTATGTAGGTACTA
ATTATTAAAAATAATTATCAAAAATATTTTTTGCGATGTTGTAAATATGAAATCTCCTTAACAACAGTATATTCA
TCTCAAATATATTAAAATGTTTAATAATATCTCAAGTGCACGAAATCAGGGTATTAGTCTAATTTGAATTTGAGT
ACATTTGGATAAAGGCAATAAAGATTGCCAATAATATATTTTTTTTTTTTTTTGAAATACCAAATTAATTATGAT
ATCTGGCAATTGATACTCACATTTGAATAAATCAGGTGTTCTAGTTATACTCTGGTAATTATTGAATCATTATTT
GAGCAAAATGCAGAATTAAAAAACAATGTGTCTACCACGTAATGCTCGCACAGATTGGCTTCCAACTTTGAATAA
TACCTAAAAGAGCAAATAAAATTTATTAGAGTTAAAAGTTTTATAAAAATATGAATATGAATGTTTTTAGGCAAG
TCAATTCTCCAACACGGTTGTAGCTAGAATATATGAATTTTGTTACGTAATAATAGATTCGGAAAATCAAAAACG
AGGTTCGTTCAATGCATTTTGTTGGTGTAATAGATATTTCTCCTATTTCTCATGTATATTGTTTGTGCAATTTAT
TGAAAGGGAAGGAAGAACAAGCAATCATATGATGTGACAACTTGACCTTCTCATATACACTCAAGCAAAATAATG
TTAAATGTTATTTGGACTAAAATATACAAAAAGGTAATGTAAAGGTAAAGTTAATATAAGCAACTACCTATGCAT
CAATATAATGTATATCGAGCCTTAAATACGATTGTTTCGAACAGGTCTGTCCCCAATTCAATGAAACAAACGAAC
AGCAATCCGTTTGTATTTATATTTACAGCTTTTTGCTTTGCCGGTAACATTTGAATCTTCATTTCTACAGTTTCA
TGTTGTTTGCCTTTAAATATTTTTCTAAGTATTTTAATAAAATTGACGAATTTAAATTTATTAATAGTATTTGAA
AAGCAAAAATTATGTAACCTTACATTTTTCATAGCAGTTAAAGATACCAATATGATTTTTAGGATAGCTCTCTAT
ACGAGCATATTTAGAAATAGTTTTATTCTTCTTTCCAATGCGACATACATTTCTACGCCGTCGTCGCTTTTAGAA
CCTTAAGTATGCATTAACGTTACTGGGACCGTAGCAATCAGTGTAGGCGGCTGACAAGTGTGATCCGCATTATCT
TTGCACTTTACTCACAGACCATTTTTTTTGTTATTCTATACGTTACATTTAATTTAAATTTTTTTAAATAATAAT
AAATAAGTTAGCGGGGAAAAATAGTTTCTAGTAAAGAGAGTGTTTATAGTGTGACAGTGTTTACGGGATCCGTAA
GTCTCCTCCCCAAGATTTTTTAAAGCAGAGGTCTACAATCATATTTAACGGAAAAATAATAGTTATATGTATATT
TTCGTGGAAGATATAACCTTTTTTTTTTAATACATCAACTATTTTTTAATACATCAACCATTTAACACTGTATTA
CAACGTTATGTATCTATACTGTTGCAGTTGTAAATACATTTCACGCCGGTATGACCCGAATGCTGTTTTCAGAAT
GGCAGCACGCAATGCTAACAACTCGCCAATAATATAAAATCTGTTCCCCTTCCGAAAGGCGGACATTTGTAATAA
ATCAAAAATTTATTTTCAATTTTTTGTGAAAGACCCTGGCGACCGTCGAGCGAAACGCAGCAATAAAACTATTAT

TTGTTGAAAAGTATAGTAATACAACGAACATTTTTTCTTAGCTTTAAGAAATTTGATTAATCTTCGATAAAAATT
ACTCTTTAATGGGTGTTCATTCAGAATTAATTTAAGATCCTTAACTTAAATGTTTTCATATAATTATACTATATA
CATATATCTTACGATATCCCAGAGCTGGACTTTATACTCTACCTGTGCATAACATCACATAACATTTTTATCTGG
TTTCTGTAGCTCAAGGCTTCCTTTACGTAAAAGTGGTGGTTATGATAAATTTCTTTATTAATCGATGAAAGTATG
AACATCTTAGAAAAAGAAATATGCACATATACATAGATGTGAATAAAAAACCTATATTTTCAG**ATCCGTTAGAAG**
CAACTAAAGACTCTCCAGTAGATGACATAATGGAAGAATTTTATTGTAACGGATATGTTGCTGACGAAAAAAATT
TATTTCAACAAGGAAAAGCATGGCATAATCCTTACTATAACTTCAATACAAATATGTGCAACAATAATGGCAACC
AGAGCAATTTAAGTTTTGGAGTTATAAATTATGGACATAACAATAGTAGTGTCGCTGCTAATCATAGTATTAACA
ACAACAATGCTTTGAATCAACATTGTTTTGAAGCCAAGTTAGGTATGCTGAACTTACCGGCCCCGTCGCCTTATT
TCAATAGCAACATGGCATTGCAGATCCCACAGCAAAATACTGCTGGAATCACAGTTGCCGCAGCAGCTGCTGCCG
CCAATATTGACTACAACAACAAAAACTCAAAAAAGGCACAGAAGCGGTATAGCAACGCAAAGTTGGATAAGTTCG
TGAGTTTATATCGAATATTATTGTTTTCATGACTAGTTGCATAGTTAATAATCATACATATGTTCATACTTATAA
GGTGCAATAGAAATTTGAAGAAGATCGGTGCGCATTACATATTTATATTTTTTTATTCTTTAGTATCGGCGTTTT
TCAGTCGATATAACATCAATGCTATTGACTAAATGTATTGAGGCGACGTTGACAATTTATCAGTAATTAATTTTA
ATTTTTATGCTACGCCATTCATTCCTTCTTTTCGCGCAATTAGAAATACCAAGTGCAGCCACATGTCTCGTATAG
CTCATCGTTCCAACAGCTGCGGTATTCACCGTTACCAACTGATAAATGATCATACGCATTACGCAAACACTTCCC
TGCGTGGACTTTACTTTGTAATTACATACTCAGTCTGCGTTAAATTTCAAAGTTGACATTGTTGTTGGCATTAAT
ACTGTATCCAATAGAGACGAAATTATACACAACTTTAAAGTAACGTAAAAGCCGAATATTACGACTGTTTGTTTG
ATGACAAAAGATATTTCGCGGCGCGGTTGTTGACGCTTTGATGGGAATATCATCGTCGTACGCCAGCTGATATAC
ATCCTTATAGAAGATTGTACCTATTCTATTCACAACCTTGTTTGGTATCGAACGTCATCCTTCAATTACAAAAAC
TTCTTTGTTAAGACGACGATTTATGAATCTACATGCAAAAAAATTGTGTATCAACAAATGTAAAAGTAAAACGCG
AATTGCATTTACATCAACTGTGGCAGATCCAGTTGATCGTTCTTATCCCTTAAAATTTTGTGCTTTATATTTTCG
CTGGTTTCTCTTTCACAATCAAAAATGATATCAGTCTGAAGCCATTCTATTTTTAATTAAAAATCCCAAATGTGT
TAAAGCTAGTACAAAAATTAAATTAAATTGTTTGGTATGGTCCATGCTGCAAAAAAAGAAAAGTGAAAGGAAAAT
TCAATGTCCGATTAAACACTTGTCTCTTGCGTATTTAATTAAATTACCCGTAATTCTGCATATTACATATTCAAA
GTAAATATAATAATACTACTCTCATATGTTTAGCGGCTAGTTGAAGTTTGAAAAAAATATTAATTTTTCGTTTGT
ATTATTTCCGGAGTTATTACAAGTGTCAATAACCAATACATACGAGTATTCGATCATTTTCAG**TCTGCAGTAAAA**
CACTGTGTTTTTTGCGAAAATAACAACGAACCGGAAGCTGTAGTGAGAAGTCATGCCGTACGCGATTCGCTAGGA
CGTGTTCTATGCCCCAAATTACGGACTTACATTTGCCCGATTTGTAAAGCCTCGGGGGGATAAGGCGCATACAGTT
AAATATTGCCCGCAAAAGCCTATTATTACAATGGAAGACGCAGTAAAAGCTGAATCTTTACGCCTAGCAAAGAAC
TTGTACTTCAAACAAGGAATGAAAGTATAA*GAAATTAGTTTTTTAAATTCTATCTTAATAGAAATTTCTTTAAAA*
AGAAAATTTTTCGTTTTTGTAAATATAGTTTCTAAGAGTCGTACAGAAAAATGAATGTACGTTAAATTATTAATT
ATTATTTACTTTTCGTTTTAGTAAATAAATTTTGTTAAGGGTAAAAAAATGTATATGAACGCACATATATGCCTT
AAAAAATGAATCGTTTAATTTAATAAACCATTCACGTATTTAAATTTCCTGCTTAATATAGATAAGTATAAATTG
AAGTGAAAGCAATATTTTTCCTTATTAAATAGCGCAGAGTTGGTTAAACTCATTTAATTTTCTGATGTGTTGAAG
TGATAATTGACACTTAAATTAAAATATTGGCATACTTCAATTGATCTCATTGAACTGTTCAATATAACATTATAC
TTATAAATTACATATCTTTACTATATTAATAACTTAACAGCTCGTAATTCATTAGCTTTGCAGCTACTCGATTTT
AATATTTTAATGTATTGTTCTTTATTGAAGTATTAACCTGCTGGTGGCGTTAAAATCGATTTAGTGGCTCATGCC
GCTTTTTATATATGAACTTATATATTGAACTTGTGCCTAAAAAATAAAATTTTTTAATACGCTCATTACAAAACT
ATCGCATACAATGACAAAATATATTCTTTTTACGAACATATAATGAATCTTTCGCTATTCATAATTGCTTATATT

TTGTATTGAGTTTAGTTTCTAGAATATCATCGCCAAGATACTCTAAAATGCTGGTATTTTTTGTGTAATTTACTT
AAGGTGGTATATATATGTAAACGTATTTGGAGTTGAATTGAAATGGTAAATTATGTAATTACCATAAATATAAGT
TTTGTTTTACATACATACGTACCATGTAACTATCTACAGCTATTGTAAATAAATAAAGAGTATTCAAACTTATCT
TTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAGT

2.9. Sequence of the region upstream of *Ccnos* gene

Underlined is the recognition site of the restriction enzyme *BglII* used for inverse PCR, the upstream region is in normal letters, in italics is the 5´UTR and in bold is part of the coding region. Sequence is in 5´ to 3´ orientation.

AGATCTTTACAATTTGTATTTCAGTTTATACATATCACATACCTGTAGCATCAGCATTAGGCTCGACCACTCTTT
CATCATTGGCATATTCAACTTCTATACGTTTCCGCAATTTTTGGGGCGAACGATAGACCCTAGAATTTATGCTTT
GTGATTCTATTTCTTGTTTTATCATTTCTGTTGTTGTTGAAATAGCCTGAAAAGCCGATGTTTGTCCTATTTTCT
CACTTTTCTCTGTAATAGTATCAGAGACACCTTTCGCAGTTTTCGATATTCCTTCAGATATTTTGGTTGCTCTCT
TTGCAATATCTGATTTACTGGCTTCGTCGAACACATCGCTGACCTTTTCTTTAATACTCCCTATTTGCTCTTTTA
AAATGTTCGAAGATTTGTGTGCTTCAGATTCAACGATGTTAAACTTTTGTCTTGCCGATTGGAGAGCTTCAGATT
GTTCTAATTTCTGCGCTTCTTCCCTGAATTTCTTTATATTTTCTTTCATTTCTTTATTCTTATCCATCTCCGATT
TGATATTATCAATGAACTGCGAAAAAAATCCCGGTCTTCTACTTGGTTGCGAATAGTAACGCTGAAAAGGTTAAA
CGGCATATTTTGCAAGTTGAAGAAACTAACAAAACCCCATACCTGATGATTTAGTCCTCGCCAGTAACCATATCC
GTGTACCATCCGGCTATATGTGCCACGAGATGCCGCCAAGATTTTGTACTTTAAGTGGATGCAAGCAAAATAACA
TGTAAGGAAGTCAACAAAACATGTTAGCATTTTAGCAGTTCCACAAATCGAGATTTTATCTTACCATTGTTAATA
AATTGCCAGTAACTTTTGCTTTAAATTTAGTTGAGAATACTAATAAACAAAATTCTAAAAAAAATTTATGTAATT
TCGTACTTATAACAGCAAAAGACAATAGTTTAAGGAACCATCACGCTGTCGAAATGTCAAAAGTTTGACAGAAAT
ATATAATAAAATATTATCGATACTAGGATCGTAATTTTCTTTTTGACAAAGCATTATCAAAAAAAGTTTGTACAA
AATATTCATGTATTTTTCAAATATCAAACATTCTGATCACCCTGTATAAGGAAGTAAAATCCGTTAGAAAGTGTA
TATGGAAATCACAGTAGAGATTTTCGTGGAAAACAACGGATTCACATTTAAAGTA*ATTTGTTAAATTCTTTCAGT*
AAGTTGGAAGCAATATATTTTGCTGCACTCAAGTGTTTTAAATCGTACCTGTTCGGTGAAATCTATGAAGATAGA
*TATTTTCATCGAGTGTATTGAAAGTACAACGTAACGGATTGTAATACTGATAGCTGTAGTAATAATT***ATGCTTGG**
AGCCAAGAGATATGCTACTTCCCGAGGGGTATGTAGAATCGGATTGCCCGTTATTTGTAAGAGTTTATTTTGGTC
ATGTCTCTCTTAAAGGATGCCGTTTCTTTTCTTGAATTTGGATCTCT