New molecular technologies to improve the Sterile Insect Technique for the Mediterranean fruitfly *Ceratitis capitata* (Diptera: Tephritidae)

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Marc Florian Schetelig

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Germany

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Referent: Prof. Dr. Ernst A. Wimmer
Korreferent: Prof. Dr. Gregor Bucher
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Meiner Familie

und

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Table of Content

1 Zusammenfassung

Schadinsekten verursachen enorme wirtschaftliche Verluste sowohl im Bereich der Viehzucht als auch in der Agarwirtschaft. Die Mittelmeerfruchtfliege *Ceratitis capitata* (Wiedemann Diptera: Tephritidae;) gehört zu den weltweit bedeutendsten und invasivsten Schädlingen, durch die Bauern jährlich mehrere Milliarden Dollar verlieren. Im letzten Jahrhundert wurden in großem Umfang Insektizide zur Schädlingsbekämpfung eingesetzt und sind auch heute noch gegen *Ceratitis* und andere Schadinsekten im Einsatz. Um den Einsatz von Insektiziden zu reduzieren und eine spezies-spezifischere Schädlingsbekämpfung zu gewährleisten, müssen existierende Methoden verbessert werden. In den letzten Jahrzehnten eröffneten biologische Ansätze neue Möglichkeiten zur Schädlingsbekämpfung. Von diesen hat sich die Sterile Insekten Technik (SIT) zu einer der ökologisch verträglichsten und erfolgreichsten Methoden zur flächendeckenden Populationskontrolle entwickelt. Sie beruht auf der Reduktion einer Schädlingspopulation durch wiederholte Massenfreisetzungen steriler Artgenossen, welche zu unfruchtbaren Paarungen führen und somit die Reduktion der Schädlingspopulation bewirken. *Ceratitis* SIT Programme sind am effektivsten, wenn ausschließlich sterile Männchen freigesetzt werden. Diese Programme bestehen aus Massenzucht, Geschlechtertrennung zur ausschließlichen Männchen-Freisetzung, Markierung zur Überwachung der Männchen nach der Freisetzung, Sterilisation, Freisetzung und Überwachung der Männchen. Obwohl die SIT schon heute erfolgreich zur Bekämpfung von *Ceratitis* eingesetzt wird, sind Verbesserungen in allen genannten Schritten nötig, um die Effizienz zu optimieren und damit die Kosten von *Ceratitis* SIT Programmen zu reduzieren.

Diese Arbeit dokumentiert die Entwicklung von transgenen Systemen zur Verbesserung der Sterilisierung, Geschlechtertrennung, Markierung und Überwachung in *Ceratitis* SIT Programmen. Erstens wurden molekulare Arbeitsweisen zur Isolierung von entwicklungsspezifischen Genen in Tephritiden erfolgreich überprüft. Zweitens wurde ein transgenes Sterilisierungssystem zur Induktion von embryospezifischer Lethalität für *Ceratitis* etabliert und erfolgreich evaluiert, das ohne die Sterilisierung mittels Gammastrahlung auskommt. Drittens wurde dieses System modifiziert, um eine Weibchen-spezifische Lethalität zu generieren, welche die Geschlechtertrennung verbessern könnte. Viertens wurden zwei Spermien-Markierungssysteme für *Ceratitis* etabliert und erfolgreich evaluiert, um die Markierung und Überwachung der freigesetzten Männchen zu verbessern. Diese transgenen, Männchen-spezifisch fluoreszierenden Marker können die bisher verwendeten problematischen bunten Stäube zur Markierung ersetzen und werden das Wissen über das Fortpflanzungsverhalten der polyandrischen Mittelmeerfruchtfliege erweitern. Fünftens wurde ein System zur Kombination oder Modifizierung von transgenen Systemen an erfolgreich getesteten genomischen Positionen etabliert. Mit Hilfe dieses Systems können in Zukunft Transgene stabilisiert und transgene Systeme zur Bekämpfung von *Ceratitis* verbessert werden. Es ist davon auszugehen, dass die hier vorgestellten transgenen Systeme oder deren Kombinationen in Zukunft die Effizienz der umweltfreundlichen SIT verbessern werden.

1 Summary

Insect pests cause enormous economic losses to livestock as well as pre- and postharvest stages of agricultural commodities. The Mediterranean fruit fly *Ceratitis capitata* (medfly; Wiedemann, Diptera: Tephritidae;) is one of the world's most destructive and invasive pest species, costing farmers billions of dollars annually. Insecticides have been used extensively during the last century and are still used to control *C. capitata* and other insect pests. However, to reduce the amount of insecticides and to increase the species-specificity of pest control, the existing tactics have to be improved. During the last decades biological approaches opened up new possibilities for insect pest management. Of these, the ecologically safe Sterile Insect Technique (SIT) developed into a powerful method for area-wide pest control. The SIT reduces the pest population by mass release of radiation-sterilized organisms, leading to infertile matings and in consequence to a decline of the pest population. Male-only releases proved to be most effective for medfly SIT programs. An SIT program for medfly includes mass-rearing, sex-separation for male-only releases, marking for monitoring, sterilization, releasing, and monitoring of male flies. Although the SIT is already successfully applied for medfly, each of these steps needs to be improved to optimize the efficiency and to reduce the costs of ongoing medfly SIT programs.

Here, I present the development of several transgenic systems to improve the steps of sterilization, sex-separation, marking, and monitoring in medfly SIT programs. First, molecular methods to isolate developmental genes were successfully practiced in Tephritid fruit flies. Second, a transgenic embryonic lethality system was established and successfully evaluated in medfly to achieve reproductive sterility without the need for radiation. Third, this system has been further modified to design a female-specific embryonic lethality system, which is intended to improve sex-separation. Fourth, two sperm marking systems were established and successfully evaluated to improve the marking and monitoring of medfly. The male-specific transgenic fluorescent markers can replace the currently used problematic dust markers in monitoring and the systems will help to increase the knowledge about reproductive biology of the polyandrous medfly. Fifth, a system was designed that makes it possible to combine or modify transgenic systems at successfully evaluated genomic sites. This can be used to stabilize transgenes and further improve the generation of transgenic medfly systems for insect pest control. I anticipate that several of the transgenic systems developed in this study and combinations thereof will increase the efficiency of the environmental-friendly SIT.

2 Introduction

2.1 Food production and pest species

The world population has tripled from about 2 billion to 6 billion people in the last century and is still growing enormously (Alexandratos, 1999). This rapid population growth is the main factor for the need of an increased amount of resources - especially water and food. In this respect, at the World Food Conference in 1996 the term ´food security´ was defined as follows: Food security exists when all people at all times have physical and economic access to sufficient food to meet their dietary needs for a productive and healthy life. Today many countries are far from achieving food security. In fact, worldwide food production systems have to change in a way that the required food can be produced and transported into regions where it is needed. Whenever possible, food should be produced in or near the area of demand. This could help to overcome the problem of unequal food distributions (Shapouri and Rosen, 1999). The possibility for agriculture and rural economy to produce sufficient quantities of food of appropriate quality and thereby decrease famine and poverty is greatly influenced by countless insect pests, besides other factors like policy and outcome in the rest of the economy (Timmer, 2000). Insect pests cause enormous economic losses to livestock as well as pre- and postharvest stages of agricultural commodities (Oerke et al., 1994). To control these pests, insecticides were used more than ten-fold in the amount and selective toxicity from 1945 up to now (Pimentel, 2007). Despite these efforts the amount of crops lost to insects has nearly doubled in the USA from 7% in 1945 to 13% at present (Pimentel et al., 1993). This example shows that pest control cannot be improved by just developing more and more chemicals, which often lead to the development of new resistances in the pest populations after a short period of time and in addition can cause long-term pollution of agricultural resources. However without pest control, losses to crops would be even more severe than they are at present (Oerke et al., 1994). Therefore different methods and strategies for pest control have been developed to account for the adaptability and variability of pest species.

2.2 Possibilities for pest control

Tactics to control or eradicate pest populations have been described for a long time in the literature, even though the incentive was often more religious or superstitiousness than scientific. 800 B.C. Homer described already the use of fire to drive locusts into the sea and refers also to the use of sulfur in fumigation. 324 B.C. the Chinese introduced predator ants in citrus trees to protect them from caterpillars and wood boring beetles (BOA, 1996). The ideas behind these traditional cases of pest control are similar to current pest control methods known as cultural, chemical or biological control. The spectrum of control methods has widened extensively during the last decade by a combination of different control methods, improvement of existing strategies and also development of new pest

control strategies. To give an insight in the wide range of present pest control possibilities, important strategies are shown in the following without making claim to be complete.

Physical and mechanical control of insects can be simple. Sticky traps, fly swatters and window screens can help to keep buildings free of many insects. In field use, a simple bagging of fruits, using nets to prevent fruit trees from being infested with flying pests or removing infested material from plantations has been proven effective. But these methods are more useful for small-scale production. In large-scale fruit production such methods are often uneconomic and less practicable (Vincent et al., 2001).

Another principle is the *cultural control*, which is besides physical and mechanical control perhaps the oldest group of pest control. It uses the modification of the growing environment to reduce the prevalence of unwanted pests. Three methods of this group are crop rotation, intercropping, and phenological asynchrony (Liebman et al., 2001). Cultural control methods have been shown as effective tools for controlling pest populations in long-term perspectives, but have often difficulties to shortly eliminate invasive species. Their efficacy depends on various natural factors, which increases the effort to manage such programs.

The *biological control* is the use of predators, parasitoids, or pathogens to suppress a pest population. Biocontrol agents include members of different life forms: vertebrates, invertebrates, fungi, and microorganisms. Insect species often become pest species when the ecological balance is interrupted by human intervention or natural events, which leads to an overgrowth of these species. The aim of releasing biocontrol agents into areas with pest infestation is the reestablishment of the ecological balance in that particular ecosystem. But this could become problematic if released biocontrol agents infest and overgrow in an ecosystem or do not work at all. Biological control programs often run for a longer time and thereby costs are increased (van Driesche and Bellows Jr., 1996).

Another variant of biological control is *birth control*, also known as *genetic control* or *autocidal control*. The principle of this strategy is suppression of a pest population by reducing its reproductive potential. Therefore reproductive sterility is induced physically, chemically, or by incorporating new and potentially deleterious genes into the genetic makeup of a pest population. A possible birth control for various key pests is the sterile insect technique (2.4; (Dyck et al., 2005a)). In that method sterility to pest insects is physically induced by ionizing radiation. It is successfully practiced in the control of several fruit fly species with the outstanding example of the Mediterranean fruit fly. However, the use of radiation itself and competitiveness problems of several species claim for improvements of this technique.

The well-known but also highly discussed *chemical control* is acting often rapid and highly reliable. Several pest species may be controlled by just a single application of chemicals. Insecticides therefore became a relatively cheap standard method to control pest species during the 20th century. Insecticides can be classified in stomach poisons, contact poisons, or fumigants. Systemic insecticides e.g. are a type of stomach poisons, which are absorbed by a plant without sick effects. Pest species feeding on

this plant ingest the insecticide and die or at least keep away from further feeding on this plant. The dichlor-diphenyl-trichlorethane (DDT) is perhaps the most famous contact poison, which was highly effective after contact or absorption, but has been banned for agricultural use since it has been supposed to cause cancer (Carson, 1962). A limited use of DDT for disease vector control is still allowed but controversial. Most conventional insecticides for chemical control are not species-specific, which could have an impact on natural enemies and the ecological balance. Other disadvantages are the development of polymorphic resistances to insecticides, which lower the insecticide effectivity and necessitate the development and use of new chemicals. In addition misuse, abuse and overuse of such insecticides created multiple resistances to several species and in some cases caused long-term environmental problems. This increased the costs and led to a widespread criticism of chemical control.

Moreover legislation and regulation of human behaviors might prevent the establishment or reduce the spread of pest populations and has become an additional aspect of IPM. Each control method has its advantages and disadvantages. In many cases rather combinations of different control methods than using a single method often leads to a successful pest control program.

2.3 Integrated pest management

By definition from the National Coalition on Integrated Pest Management, integrated pest management (IPM) is a sustainable approach to managing pests by combining biological, cultural, physical and chemical tools in a way that minimizes economic, health and environmental risks. IPM requires knowledge about ecological principles, pest life history, and population dynamics. In the 1950s a strategy of combined pest management evaluations, decisions, and controls was first shown to improve the pest management in agriculture (Stern et al., 1959). Today the concept of IPM is practiced worldwide. IPM shows its success in more and more projects and reduces the use of pesticides (Brower, 2002). IPM works in small environments (Brenner et al., 2003), but is also used in area-wide pest management programs (Vreysen et al., 2007). To set up a successful IPM program, several guidelines for various circumstances have been prepared, but in general some basic steps of integrated pest control remain always the same:

- Definition of an action threshold: An action threshold is a point at which environmental conditions or pest populations indicate that pest control action must be taken. This threshold is set to exclude needless pest control to sporadically appearing pests.
- Monitoring and identification of pest species: Many detected organisms are innocuous or even beneficial for the economic balance and not every insect or living organism requires control. Pest species and closely related species with different pest potentials have to be discriminated and

identified before pest control. Therefore, IPM programs consist of monitoring and identification of pest species, which help in combination with the action threshold to find the best control methods.

- Prevention: First of all, IPM programs try to keep the crops free from pests by managing the whole production processes with effective and cost-efficient methods that are at low or no risk for environment and communities. E.g., first steps can be crop rotation or sanitation to avoid a first infestation of pest species.
- Control: A pest control program is started, once the action threshold, monitoring, and identification data show its need and prevention methods are no longer available or effective. The whole spectrum of pest control methods is then taken into account to find the most effective and riskless control method(s). Non-specific controls like some pesticide sprayings are always used as a last resort.

To further improve pest control, IPM programs are searching for most environmental-friendly methods to reach acceptable pest status. In this respect, one strategy has become a powerful tool for area-wide IPM programs of a number of pest species over the last 50 years: the sterile insect technique.

2.4 Sterile insect technique

The sterile insect technique (SIT) is an environmental-friendly control method also referred to as a kind of birth control (2.2). In principle, a large number of reproductively sterile male insects are released into a wild population of the same species so that they mate with wild females and thereby block their reproductive capacity (Fig. 1) (Knipling, 1955).

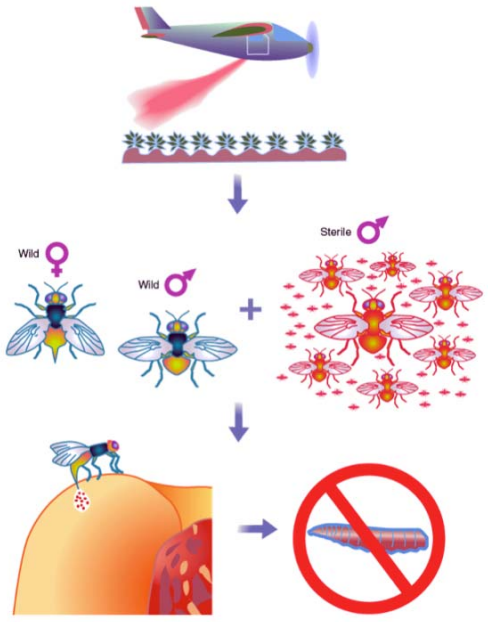

Figure 1. Priciple of the SIT. (from (Wimmer, 2005))

A successful use of SIT for pest control requires a sufficiently high overflooding ratio of mass reared, sterilized, and released males, which are able to compete with wild males and to mate with wild females. There are several different means to implement SIT in area-wide IPM (AW-IPM) programs, known as eradication, suppression, containment, and prevention (Knipling, 1979).

First of all, the eradication strategy is defined as an application of phytosanitary measures to eliminate a pest from an area (FAO, 2005). It was first shown to be successfully eliminating the New World Screwworm *Cochliomyia hominivorax* from the island of Curacao (Baumhover et al., 1955). This eradication technique was then used for more than 40 years to eliminate the screwworm from the USA, Mexico, and Central America up to Panama (Vargas-Teran et al., 2005). Eradication programs have the ability to eliminate complete pest populations species-specifically and lead to a reduction in the use of insecticides implying a long-term benefit for the environment. In addition, eradication allows the establishment and declaration of "pest free areas", which can permit access to otherwise closed export markets (Malavasi et al., 1994). In the context of AW-IPM programs the eradication strategy is often used during the last phase of the program and mainly for eliminating an established pest population (e.g. the tsetse fly *Glossina austeni* in Zanzibar) or to eliminate outbreaks of invasive species before their full establishment (e.g. New World Screwworm from Libya (Krafsur and Lindquist, 1996), painted apple moth from New Zealand (Suckling, 2003) or the Mediterranean fruit fly from the Los Angeles Basin in 1980-1996).

Second, the suppression strategy is an alternative to the eradication. By definition suppression is the application of phytosanitary measures in an infested area to reduce pest populations (FAO, 2005). Suppression strategies are used to maintain the pest population below defined levels to ensure the economic health. SIT strategies and especially long-duration suppression strategies as a part of AW-IPM programs have become more popular since several premises and views have been changed. Thus, increased restrictions on the use of insecticides in combination with the improved excess to mass-reared key pest species have significantly improved the cost-efficiency of SIT as part of AW-IPM programs (Caceres et al., 2004; Matteson, 1995). In addition, an increasing demand of organic products leads to an expansion in using environmental-friendly pest control strategies like the SIT (Economist, 2001), which can replace the use of several chemicals. SIT suppression programs for pest species are less complex and management intensive than eradication programs and can be achieved more quickly and cost-effective in the initial years (Mumford, 2005). However, suppression strategies require continuing releases of sterile insects to maintain a low population level. Suppression programs were successfully run for the oriental fruit fly in Thailand (Enkerlin, 2003), the codling moth in British Columbia, Canada (Calkins et al., 2000), and the Mediterranean fruit fly in Israel and Jordan (Rössler et al., 2000), in Madeira (Pereira et al., 2000) and in South Africa (Barnes et al., 2004).

Third, the *containment* or *prevention strategy* is defined as an application of phytosanitary measures to prevent spread of pest in and around an infested area or to avoid the introduction of a pest into pest free areas, respectively (FAO, 2005). Examples for *containment strategies* are the Queensland and

Mediterranean fruit fly programs in Australia (Jessup et al., 2007), the New World screwworm program in Panama or the Mediterranean fruit fly program in Guatemala-Mexico and Peru-Chile. All these programs avoid the establishment of invading exotic pests or consolidate the progress made in an ongoing eradication program. Containment programs are able to protect neighboring pest free areas, which can be expanded gradually. Achieving an intensive cooperation as well as the disruption of trade and free movement of commodities between infested and non-infested areas is absolutely necessary, but at the same time displays two major problems to solve in containment programs.

In contrast, preventional programs are carried out in pest free areas to maintain a pest free status. This *preventional strategy* was described by Edward F. Knipling as the probably most cost-effective variant of using SIT (Knipling, 1979). A preventional strategy is useful if an area is under constant threat of pest invasions, which can be prevented from their development and establishment by releasing sterilized males of the same species. E.g. preventional releases of sterile melon flies are applied in Okinawa (Japan) to avoid reestablishment of melon fly coming from Taiwan (Kuba et al., 1996). After technically, politically and environmentally successful eradications in the year 1996, the probably most visible preventional medfly SIT pest programs were started to prevent the areas of the Los Angeles Basin (California, USA) and Tampa-Miami (Florida, USA) from new Mediterranean fruit fly infestations (Hendrichs et al., 2002). For all parties of a pest management program there seems to be no more biologically and economically efficacious, environment-friendly and cheaper method to prevent and exclude medfly from these areas (CDFA, 2000).

Several examples described above showed that it is possible to run a successful AW-IPM program including a SIT strategy, but these programs have to be well planned and various important program phases have to be managed. The anticipated phases for a SIT program are as follows:

Pre-intervention phase. Data on the distribution and the population dynamics of the target species have to be collected (Ito and Yamamura, 2005; Vreysen, 2005). In addition, an infrastructure for mass-rearing, sterilization, packing, releasing, and quarantine has to be established and public relations work has to be started (Dyck et al., 2005b).

Population suppression phase. Due to the required overflooding ratios in SIT programs, SIT is most effective on relatively low-density populations. Prior to any releasing action from within SIT programs, the population has to be decreased by other pest control methods (2.2), if not already at a low level as a result of climatic conditions (changes summer-winter) or natural decline (changes due to the life-cycle of the target species) (Mangan, 2005).

Release phase. Repeated releases of sterile insects over the infested area are carried out to reduce target populations to an acceptable level (suppression strategy), to eradicate target populations (eradication strategy), or to avoid new pest infestations (containment and preventional strategy) (Dowell et al., 2005).

Maintenance and verification phase. If the aim of a SIT program was the eradication of a pest species, a pest free status is confirmed and preserved by permanent implementation of monitoring and quarantine activities. Once a low prevalent status of a target area has been achieved, suppression or containment and preventional releases are further carried out (Barclay et al., 2005).

Beside the fact that SIT is a successfully practiced component of area-wide pest management programs, limitations exist in various steps of the described SIT program, which can be optimized to widen the possibilities for this environment-friendly pest control technique.

2.5 Improving SIT

The field of research on improving the SIT is enormous. In general scientists are working on SIT improvements for nearly every step in various pest species (Robinson and Hendrichs, 2005). This research helps to optimize the cost-efficiency of SIT programs and is also needed to expand the use of the technique for new key pest species. In the following, technical improvements to the SIT are shown for some steps.

A first step of SIT, which is constantly under improvement, is sex separation. During the mass-rearing process of several insects, males and females have to be separated for male-only releases, because it has been shown that these are most effective (McInnis et al., 1994; Rendon et al., 2004; Rendon et al., 2000). This can be labor-intensive if external morphology and hand sorting are used for sex-separation like practiced for *Glossina austeni*, the tsetse fly. New knowledge about the variation in the developmental rate of tsetse sexes led to a sex separation system based on the timing of adult emergence, which eliminated the laborious hand-sorting (Opiyo et al., 1999; Opiyo et al., 2000). In the Oriental fruit fly *Bactrocera dorsalis* (McInnis et al., 2005) and the melon fly *Bactrocera cucurbitae* (McInnis et al., 2004) pupal color separation systems were developed and are used now to separate the sexes. In the Mediterranean fruit fly several different markers are available, but most of them are not ideal for large-scale SIT applications. Current medfly genetic sexing strains (GSSs) carry two mutations, the *white pupae* (*wp*; (Rössler, 1979)) and the *temperature-sensitive lethal* (*tsl*; (Franz et al., 1994)). The big advantage of these GSSs over marker-only sexing strains in medfly is the simple sexing by heatshock without the need for separation. Via heatshock all females of this GSSs are killed during early embryogenesis, but at the same time males are maintained. Thus, the mass rearing of medfly, with a weakly production of 3500 million males, was tremendously improved by the introduction of GSSs (Franz, 2005). Nevertheless, only a maximum of 70% of total males survive during the sexing procedure in GSSs due to the mutations in the optimized GSS *Vienna 8* (Gerald Franz, personal communication). Transgenic sexing systems based on tetracycline repression systems were first developed and tested in *Drosophila melanogaster* (*D. melanogaster*) (Heinrich and Scott, 2000; Thomas et al., 2000) and recently a transgenic sexing system for medfly was generated (Fu et al., 2007). With this system it is possible to kill 99,9% of medfly females by conditional lethality

combined with alternative, sex-specific splicing. On the one hand this system might be able to produce more viable males than existing GSSs, but on the other hand the majority of the female lethality occurs at late larval stages and the strains are not completely sterile in matings with wild females. The transgenic medfly strains have so far not been tested in larger scales and/or for any fitness penalties. The constant genetic improvements demonstrate the progress in this field, but there is still a high potential to further increase the efficiency of existing sexing systems or to develop new ones - even for key pest species like the medfly.

Once a species is sexed, the next problematic step has to be carried out: the sterilization. A sterile insect in the sense of SIT is defined as "an insect that, as a result of an appropriate treatment, is unable to produce viable offspring" (FAO, 2005). In the beginning of SIT programs chemosterilants and irradiation were used to sterilize insects. The efficiencies of both were similar (Flint et al., 1975), but since most of the chemosterilants, which are partially introduced into the environment with the release of the insects are carcinogenic, teratogenic, and/or mutagenic, the exposure of insect to ionizing radiation has become the method of choice for most species. Irradiators using cobalt-60 or caesium-137 have been designed to keep the radiation exposure and doses to workers "as low as reasonable achievable" meaning that there will always be constant low exposure in the range of recommended dose limits and a residual risk (Bakri et al., 2005). Other difficulties are insect species like some Lepidoptera, which produce progeny even after irradiation doses of 500 Gy (in comparison 60 Gy are sufficient to guarantee 100% sterility for screwworm). The existence of such species led to the development of genetic methods to induce sterility. A first approach to cause reproductive sterility by transgene-based embryonic lethality without the need of radiation was successfully shown in the non-pest insect *D. melanogaster* (Horn and Wimmer, 2003). The system is based on the transmission of a transgene combination that causes embryo-specific lethality in the progeny. However, prior to possible applications such a system has to be established or transferred to key pest insects. In the medfly, transgenic strains were recently produced, which also lead to lethality when mating transgenic males to wild type females (Gong et al., 2005). But the majority of the lethality for this medfly strains occur at late larval stages compared to embryonic lethality in irradiation-sterilized strains or the described transgenic *D. melanogaster* strains. If such systems could be further improved for medfly or other species, costs will be reduced and the rearing safety for workers and environment will increase (no radiation; no constant threats from accidental fly releases from rearing facilities because of using dominant lethal strains).

After the flies are sterilized, there is the issue of field monitoring during the release and maintenance phases of an SIT program. For this, effective methods to attract and trap insects of both sexes are needed and various trapping methods have been developed. Once insects are trapped, these data are used to calculate the ratio of released to wild insects and indirectly the success of the control program (Vreysen, 2005). During these monitoring process it is important to easily recognize and differentiate released insects from wild ones. For this reason, released medfly males are dusted with fluorescent

powders before release (Parker, 2005) and therefore can be distinguished from wild counterparts in field traps afterwards. This technique of marking is expensive, error-prone and labor intensive (Robinson and Hendrichs, 2005). Morphological markers can be an alternative to fluorescent dusts, if they do not reduce the competitiveness of the insects. For medfly a phenotypic mutant could be isolated, which was competitive in mass-rearing and field cage competition tests (Niyazi et al., 2005). Several other applicable marking techniques are discussed for SIT (Hagler and Jackson, 2001). Among these methods, the marking of insects with genetically engineered proteins has enormous potential to improve field monitoring. For two mosquito species, *Anopheles gambiae* (*A. gambiae*) and *Aedes aegypti*, genetically engineered fluorescent marking was established and can now be used for monitoring (Catteruccia et al., 2005; Smith et al., 2007). The fluorescence was even limited to the testes of males and could therefore be used to differentiate between released and wild type males and to identify the mating status of the wild type females (Smith et al., 2007). Up to now such genetic systems have been missing for the key pest species medfly.

In several steps of SIT, first transgenic approaches have shown the potentials of generating systems with improved characteristics. But they have to be further modified and evaluated, transferred to key pest species, or designed new to widen their possibilities for AW-IPM programs. In addition, the safety of transgenes has to be considered, when thinking about an application of transgenes in SIT programs.

2.6 Research objectives

To start transgenic work, molecular techniques to isolate genes from the Tephritid species *Ceratitis capitata* (*C. capitata*; medfly) and *Anastrepha suspensa* (*A. suspensa*; caribfly) were to be practiced. Therefore the developmentally conserved *orthodenticle* genes from both species should be isolated and the expression patterns of their mRNAs visualized (see 3.1).

Thereafter, new transgenic systems should be developed in the Mediterranean fruit fly *C. capitata* to improve different aspects of the SIT. Two systems should be developed:

i) A conditional embryonic lethal system inheriting reproductive sterility without the need of radiation (3.2, 3.3, and 3.4).

ii) A sperm marking system for inherited marking and improved monitoring of medfly in SIT programs (3.6)

To generate an embryonic lethality system for medfly, a system known from *D. melanogaster* (Horn and Wimmer, 2003) should be transferred to medfly (see 3.2 and 3.3). In the case, a direct transfer would not work, early embryonic active genes were to be isolated from medfly (see 3.2 and 3.4). The putative promoter regions of these genes could then be used to drive a transgene combination that causes lethality. Embryonic lethality strains were then to be tested in laboratory and field cage studies to evaluate the system for functionality, competitiveness, and fitness. Once a functional embryonic

lethality system had been developed, it might be combined with a female-specific spliced intron from the medfly *transformer* gene to generate a transgenic sexing system in medfly (see 3.5), which might kill female embryos during the mass-rearing process, but leaving viable males for releases.

To generate a sperm marking system in medfly, the spermatogenesis-specifically expressed *β2-tubulin* gene and its putative promoter were to be isolated from medfly. The promoter was then to be fused to fluorescent proteins. After integration of these fusion constructs into the genome of medfly by *piggyBac* transposition, a spermatogenesis-specific expression of fluorescent proteins was expected. Such marked medfly strains, if tested competitive to non-transgenic flies, could become powerful tools to improve the monitoring procedure during SIT programs and to get new knowledge about reproductive biology in medfly.

To possibly modify the genomic situation of characterized strains by site-specific integration, all generated systems were to carry targetable integration sites. In this respect, the phiC31-mediated site-specific integration should be established in medfly and then used for scenarios like increasing the stability of transgenes or combining different genetic systems at a characterized genomic position (see 3.7).

3 Results

Every chapter within the results starts with a one-page description of:

- the main aim of the particular manuscript in the context of the complete thesis
- the authors and their contributions to the practical work, and
- the status of the manuscript.

3.1 Plasticity in mRNA expression and localization of *orthodenticle* within higher Diptera

In this part, molecular techniques for the isolation of developmental genes were to be practiced on the Tephritid species *C. capitata* and *A. suspensa*. Therefore, the developmentally conserved *orthodenticle* genes were isolated. Their expression patterns were detected by whole mount *in-situ* hybridization and displayed a surprisingly different distribution and localization of *otd* mRNAs in ovaries and during embryogenesis compared to each other and to *D. melanogaster*.

Schetelig, M. F.◐, Schmid, B. G. M.◐, Zimowska, G., and Wimmer, E. A.

◐ = co-first authors

Author contributions to the practical work:

Schetelig, M. F.◐: Isolation of *A. suspensa otd*; preparation of *C. capitata* ovaries, *C. capitata* embryos, and *A. suspensa* ovaries; whole mount *in-situ* hybridizations to embryos and ovaries

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Plasticity in mRNA expression and localization of *orthodenticle* within higher Diptera

Marc F. Schetelig[*◐], Bernhard G. M. Schmid[*◐], Grazyna Zimowska[†] and Ernst A. Wimmer[*§]

[*] Department of Developmental Biology, Göttingen Center for Molecular Biosciences, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, Georg-August-University Göttingen, GZMB, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany.
[†] USDA/ARS, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, Florida, USA.

§ To whom correspondence should be addressed. E-mail: ewimmer@gwdg.de; Tel.: ++49-551-392889; Fax: ++49-551-395416

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Running head: Maternal expression of *otd* in Diptera

Abstract

***orthodenticle* (*otd*) genes are found throughout the animal kingdom and encode well studied homeodomain transcription factors that share conserved functions in cephalization, head segmentation, brain patterning and the differentiation of photoreceptors. Otd proteins have been proposed as ancestral key players in anterior determination despite a high level of variation in gene expression at early developmental stages: *otd* is expressed strictly zygotically in the dipteran *Drosophila melanogaster*, while *otd1* contributes maternally to the embryo in the coleopteran *Tribolium castaneum* and shows localization of maternal *otd1* mRNA to the anterior and posterior pole of the oocyte in the hymopteran *Nasonia vitripennis*. Here we demonstrate that such changes in *otd* mRNA expression and localization do not need to represent large phylogenetic distances but can occur even within closely related taxa. We show maternal *otd* expression in the medfly *Ceratitis capitata* and maternally localized *otd* mRNA in the caribfly *Anastrepha suspensa*, two cyclorrhaphan species closely related to *Drosophila*. This indicates considerable plasticity in expression and mRNA localization of key developmetal genes even within short evolutionary distances.**

Keywords: *Anastrepha suspensa*, *bicoid*, *Ceratitis capitata*, head development, maternal expression

Introduction

The gene *orthodenticle* (*otd*) encodes a paired class homeodomain transcription factor (Treisman et al. 1992). Since its characterization in *Drosophila melanogaster* (Finkelstein et al. 1990), *otd* homologs have been found in a great number of animal species, ranging from various arthropods to vertebrates as well as diploblasts: *Tribolium castaneum* (Schröder 2003), *Nasonia vitripennis* (Lynch et al. 2006), *Parhyale hawaiensis* (Browne et al. 2006), *Euscorpius flavicaudis* and *Tegenaria saeva* (Simonnet et al. 2006), *Mus musculus* (Simeone et al. 1993), *Xenopus laevis* (Andreazzoli et al. 1997), and *Hydra vulgaris* (Smith et al. 1999). Because *otd* homologs play major roles in cephalization, head segmentation, brain regionalization and photoreceptor development across such diverse species, they have been considered as ancestral key players with a high level of functional conservation (Acampora et al. 1998; Andreazzoli et al. 1997; Chen et al. 1997; Cohen and Jürgens 1989; Finkelstein and Perrimon 1990; Schinko et al. 2008; Vandendries et al. 1996).

In *Drosophila*, *otd* expression is exclusively zygotic and under the control of the maternally provided anterior determinant Bicoid (Bcd (Gao and Finkelstein 1998)). Apart from *Drosophila*, *bcd* has so far only been found in cyclorrhaphan dipterans (Stauber et al. 2002). It appears to be absent in lower dipteran species as well as other holometabolous insects like the hymenopteran *Nasonia* and the coleopteran *Tribolium* (Brown et al. 2001). Interestingly, there is evidence that *bcd* has evolved from a recent *Hox3* duplication that has occurred at the basis of the cyclorrhaphan flies (McGregor 2005; Stauber et al. 2002).

However, many of the species that do not have *bcd* activity undergo long-germ development (e.g. *Nasonia*) or have a syncytial blastoderm that provides patterning information for at least the most anterior segments and embryonic tissues (e.g. *Tribolium*) (Brown et al. 1994). Such modes of development clearly profit from an early anterior determinant.

In the coleopteran *Tribolium*, anterior patterning depends highly on the *otd* homolog *otd1* that is maternally expressed during oogenesis and the mRNA is ubiquitously distributed in the oocyte (Schröder 2003). This together with the fact that Otd and Bcd show similar DNA-binding properties has supported the idea that *otd* acts as an ancestral anterior determinant whose function has been taken over by *bcd* during its rise in cyclorrhaphan flies (Lynch and Desplan 2003). In the hymenopteran *Nasonia*, *otd1* is maternally expressed, the mRNA localized anteriorly as well as posteriorly in the oocyte, and Otd1 provides crucial patterning information at both poles in the early wasp embryo (Lynch et al. 2006).

Therefore, the early patterning mechanisms in insects appear to be quite plastic. Even the way conserved players like *orthodenticle* are expressed and perform their function is highly variable among different insect orders: maternal (*Tribolium*, *Nasonia*) versus strict zygotic (*Drosophila*) expression, localized (*Nasonia*) versus non localized (*Tribolium*) mRNA. In this study, we take a close look at the expression of *otd* homologues in two Tephritid species, which are closely related to the Drosophilids:

the medfly *Ceratitis capitata* and the caribfly *Anastrepha suspensa*. We can show that changes in maternal versus zygotic *otd* expression as well as mRNA localization can happen at short evolutionary distances and might not be characteristic of insect orders or suborders.

Materials and Methods

Fly stocks

Wildtype *Drosophila melanogaster*, *Ceratitis capitata* and *Anastrepha suspensa* lines were maintained under standard rearing conditions (Handler and Harrell 2001; Roberts 1986; Saul 1982).

Isolation of *orthodenticle* genes from medfly and caribfly

Based on protein alignments of *Drosophila melanogaster* and *Tribolium castaneum* OTD degenerate primers were designed to isolate *otd* from medfly and caribfly. From both species polyA$^+$ mRNA was extracted from embryos using the Micro Poly(A) Pure Kit (Ambion, Austin). Three cDNA pools were generated for medfly and caribfly using the respective polyA$^+$ mRNA pool: i) a double-strand cDNA using BD SMART PCR cDNA Synthesis Kit (BD Biosciences, Heidelberg); ii) a 5´ single strand cDNA for RACE and iii) a 3´ single strand cDNA for RACE using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Heidelberg). By PCRs (3 min at 94 °C; 35 cycles of 1.30 min at 94 °C, 2 min at 50 °C, 1 min at 68 °C; and 10 min at 68°C) using degenerate primers (fw_QRRERTTFT (CAGMGGMGGGARMGIACIACITTYAC) and rev_QVWFKNRRA (GCCCKCCKRTTYTTRAACCAIACYTG), a 160 bp fragment of medfly *otd* (*Cc-otd*) from the medfly double strand cDNA pool and a 160 bp fragment of caribfly *otd* (*As-otd*) using the caribfly double strand cDNA pool was isolated. The 5´ and 3´ ends of the medfly *otd* or caribfly *otd* were isolated by RACE PCRs (5 cycles of 5 sec at 94 °C, 3 min at 72°C; 5 cycles of 5 sec at 94 °C, 10 sec at 70°C, 3 min at 72°C; 22 cycles of 5 sec at 94 °C, 10 sec at 68 °C, 3 min at 72 °C; and 10 min at 72°C) using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Heidelberg), 5´ and 3´ single strand cDNA pools from medfly or caribfly, the gene-specific primers CC-OTDrev (GTGAATGTTGTACGCTCACGTCTCTGCTTGCGGG) or AS-OTDrev (GCGACCTCCTCGCGCATAAAGATGTCCG) for the 5´ end, and CC-OTDfw (GGGGTGTTAACACCCGCAAGCAGAGACGTGAGCG) or AS-OTDfw (GCGCGCCCAATTGGATGTACTCGAATCGC) for the 3´ end, respectively. Isolated fragments were cloned into pCRII vectors (Invitrogen, Karlsruhe) and sequenced. The plasmids p*CRII-Ccotd5g5* and p*CRII-Ccotd3g3*, containing a 1681 bp 5´ and a 2254 bp 3´ RACE fragment were sequenced and identified by BLAST algorithms as medfly *otd* homologous sequences, respectively. The plasmids p*CRII-Asotd55* and p*CRII-Asotd33*, containing a 1347 bp 5´ and a 2255 bp 3´ RACE fragment, were sequenced and identified by BLAST algorithms as caribfly *otd* homologous sequences, respectively.

cDNA sequences of caribfly and medfly *otd* are shown in GenBank (Accession nos.: *As-otd*, EU443100; *Cc-otd*, EU443099).

In-situ hybridizations

WMISH with RNA probes to embryos were performed as described (Davis et al. 2001) with the exception of manually devitellinization of preblastodermal stages of *Anastrepha suspensa* embryos. RNA antisense probes were prepared by *in-vitro* transcription with the DIG-RNA-Labeling Kit (Roche, Mannheim). The plasmids p*BSK-Dmotd* (Finkelstein et al. 1990), p*CRII-Ccotd3g3* or p*CRII-Asotd33* were linearized with *Kpn*I, *Spe*I or *Not*I and transcribed with T3, T7 or Sp6 RNA polymerase, respectively. WMISH to ovaries were performed as described (Tautz and Pfeifle 1989) using the paraformaldehyde fixation step and RNA probes prepared as above.

Results

Isolation and sequence analysis of *otd* from *Ceratitis capitata* and *Anastrepha suspensa*

To compare *orthodenticle*-related genes from medfly and caribfly with *Drosophila melanogaster orthodenticle,* we isolated *otd* genes by degenerate primer PCRs on embryonic cDNA pools. The sequence similarity of known *otd* homologs from *Drosophila*, *Tribolium* or vertebrates is known to be limited to the homeodomain region (Li et al. 1996). Thus, for both medfly and caribfly, degenerate primers were chosen to amplify 53 amino acids of the homeodomain. 160 bp fragments from medfly and from caribfly were subcloned, sequenced and the obtained sequence was used for the subsequent isolation of medfly *otd* (*Cc-otd*) and caribfly *otd* (*As-otd*) by RACE PCRs. The medfly and caribfly sequences predicted proteins of 505 (CC-OTD) and 506 (AS-OTD) amino acids, respectively. The predicted protein products from *Cc-otd* and *As-otd* contained homeodomains identical or highly similar to the homeodomain of *Drosophila* OTD.

otd expression in ovaries

To detect the expression of *otd* during oogenesis of *Drosophila*, medfly and caribfly, we did whole mount *in situ* hybridization (WMISH) to ovarioles dissected from unmated females. In *Drosophila* we could not detect *otd* transcripts in oogenesis (Fig. 2A-C) and thereby confirmed the lack of maternal *otd* expression (Finkelstein and Perrimon 1990). In contrast, *Cc-otd* was maternally expressed starting at oogenesis stage eleven (Fig. 2D,E): *Cc-otd* mRNA was located in the nurse cell cytoplasm and later in the oocyte. At stage 13 *Cc-otd* mRNA was distributed all over the oocyte, but was not present in nurse or follicle cells (Fig. 2F). In caribfly *As-otd* mRNA was detected first at stage eleven in the nurse cell cytoplasm (Fig. 2G,H) and was not detected in nurse cell nuclei or follicle cells. During and after nurse cell-dumping, *As-otd* mRNA was also detected in the oocyte (Fig. 2H,I), but localized at the anterior pole (Fig. 2J), which was in contrast to the uniform distribution of *Cc-otd* in the medfly

oocyte (Fig. 2F). In summary, *otd* transcripts were not detected during oogenesis in *Drosophila melanogaster*, strongly contributed maternally in medfly and caribfly, but localized only in caribfly.

otd expression during embryogenesis

To further check the *otd* expression during embryogenesis, WMISH to *Drosophila*, medfly and caribfly embryos was performed. Embryos at preblastoderm stages (Fig. 3A,F,K) showed an *otd* distribution comparable to the late oogenesis stages (Fig. 2C,F,I): *Drosophila otd* mRNA was not detected (Fig. 3A), *Cc-otd* mRNA was distributed ubiquitously with a slightly stronger signal in the middle of the embryo (Fig. 3F), and *As-otd* mRNA was localized to the anterior tip (Fig. 3K). These differences in preblastoderm stages are likely resulting from the maternal distribution of *otd*, which were already visible in similar patterns in the oocytes (Fig. 2).

At the syncytial blastoderm stage of *Drosophila*, *otd* expression is covering roughly the anterior most 25% of the embryo (Fig. 3B). In comparison, *Cc-otd* was uniformly expressed in the anterior half of the embryo (Fig. 3G). *As-otd* mRNA was distributed gradient-like with the highest concentration at the anterior pole of the embryo (Fig. 3L). These differences in syncytial blastoderm *otd* expression might be more related to the zygotic expression of *otd*, even though in caribfly the detection of a mixed maternal and zygotic *otd* expression is possible.

From the cellularized blastoderm stage onward, *otd* expression looked very similar in all three species: at the cellularized blastoderm stages the anterior cap was narrowed from anterior and posterior resulting in a defined broad head stripe (Fig. 3C,H,M); *otd* was detected in similar patterns during germ band elongation in the head lobes and the developing central nervous system (Fig. 3D,I,N); during head involution and dorsal closure, similar *otd* expression was detected in the head and the germ band (Fig. 3E,J,O).

Discussion

Variance and conservation of *otd* expression in higher Diptera

The differences in *otd* mRNA expression and localization occur along with other differences like prolonged syncytial development and missing elongation of the nuclei during the slow phase of cellularization in early development of *Ceratitis* embryos compared to *Drosophila* (Schetelig et al. 2007). The high degree of similarity in late *otd* expression patterns is consistent with previous findings that *otd* function is highly conserved in the well-described processes of head segmentation, brain regionalization, and photoreceptor differentiation (Acampora et al. 1998; Chen et al. 1997; Cohen and Jürgens 1990; Finkelstein and Perrimon 1990; Vandendries et al. 1996).

Variance in mRNA expression and localization within short evolutionary distances

Variation of maternally expressed and localized *otd1* in *Nasonia*, maternally expressed but ubiquitously distributed *otd1* in *Tribolium*, or strictly zygotically expressed *otd* in *Drosophila* was shown for species belonging to different insect orders (Fig. 1). Concordant with these findings on *otd* homologs, other developmental genes show analogous variations in mRNA localization modes over large evolutionary distances: e.g. *Nasonia giant* (Brent et al. 2007) and *Tribolium eagle* (Bucher et al. 2005) are expressed maternally and localized at the anterior pole of the embryo, whereas *Drosophila giant* is a zygotically expressed gap gene and *Drosophila eagle* is inactive in the early blastoderm and expressed later in a subset of developing neuroblasts.

Here we present a similar varibility for *otd* homologs within higher Dipterans: maternal and localized *Anastrepha otd* mRNA, maternal but not localized *Ceratitis otd* mRNA, and the strictly zygotic *Drosophila otd* expression. This indicates that dramatic changes in gene expression and mRNA localization of developmental genes can occur even within closely related species. Thus we conclude that early developmental expression patterns and the existence of mRNA localization cannot serve as phylogenetic character states. Instead, they are part of a rather plastic system that can easily change. During evolution, it seems not to require much effort to change gene expression from zygotic to maternal or vice versa and to actually localize mRNA. This might be interesting in respect to *bcd* evolution for which it has been strongly discussed, how difficult such evolutionary changes might be: i.e. for a zygotic gene (*Hox3*) to become maternally expressed and its mRNA localized (McGregor 2005). This study shows that such variability exists within short evolutionary distances and might not be as difficult to achieve as originally thought.

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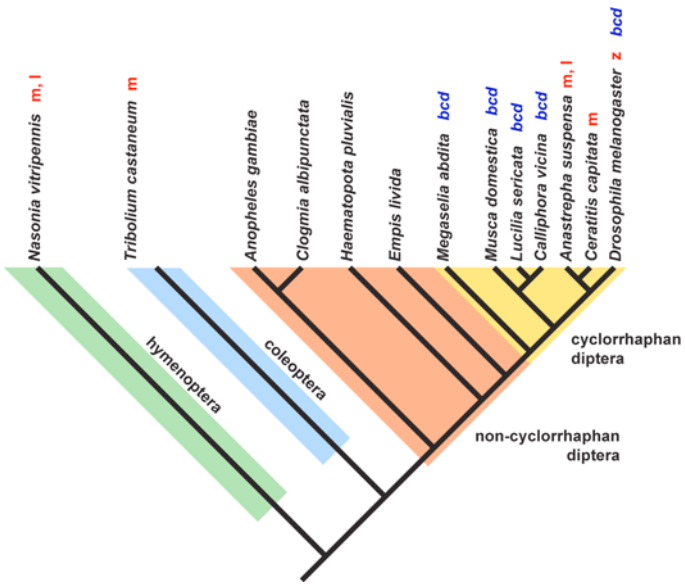

Figure 1. Phylogeny of dipteran, coleopteran, and hymenopteran species of interest

Zygotic (**z**) or maternal (**m**) expression, and oocyte localization (**l**) of *otd* mRNA are indicated on a phylogenetic tree based on NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy). *bicoid* (***bcd***) genes that act as anterior determinants have only been identified in cyclorrhaphan Diptera (McGregor 2005).

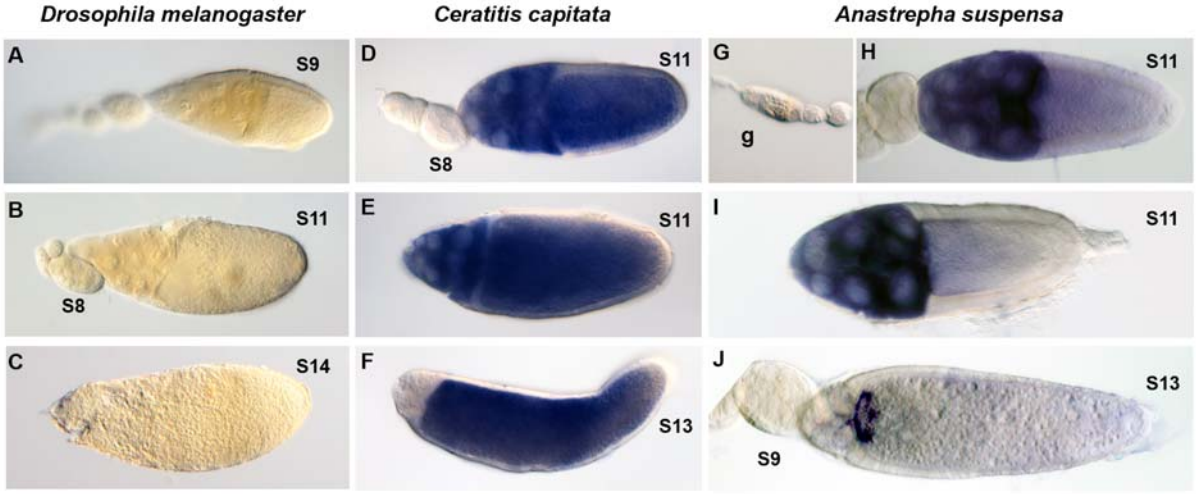

Figure 2. *otd* expression in ovaries

otd expression in ovaries from unmated wildtype females is shown by WMISH: *Drosophila melanogaster* (A-C), medfly (D-F), and caribfly (G-I). Indicated stages of oogenesis are as described (King 1970): g = germarium. For details see main text.

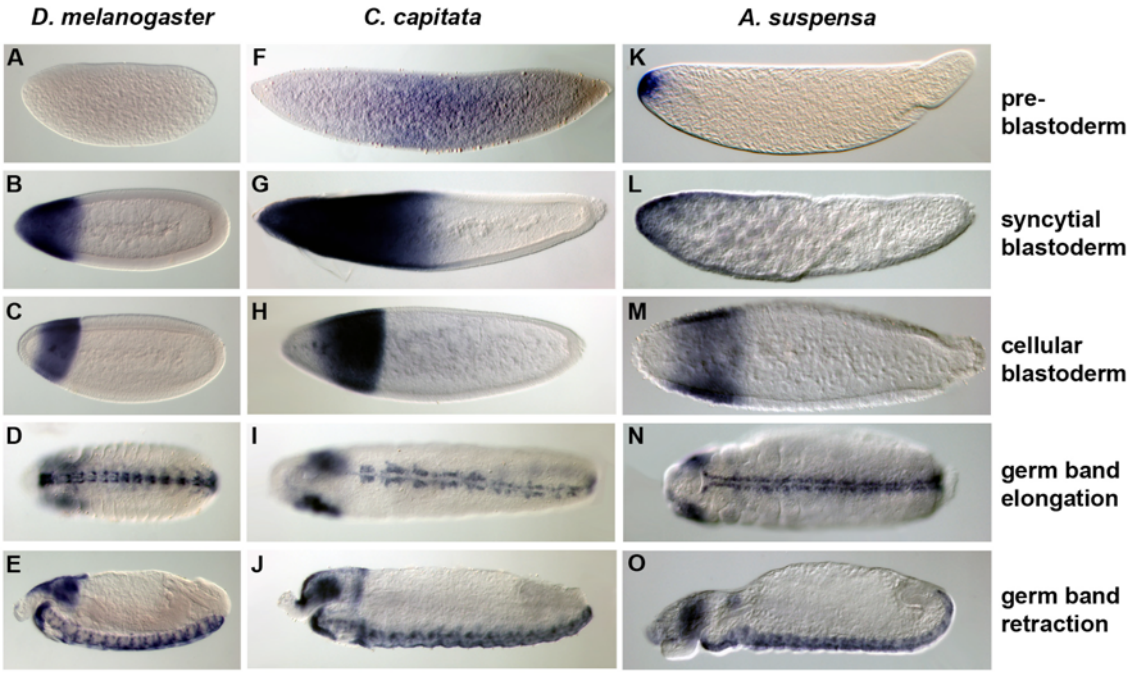

Figure 3. *otd* expression during embryogenesis

otd expression during embryogenesis is shown by WMISH: *Drosophila melanogaster* (A-E), *Ceratitis capitata* (F-J), and *Anastrepha suspensa* (K-O). Indicated stages of embryogenesis are as described (Campos-Ortega and Hartenstein 1997). For details see main text. *Anastrepha suspensa* embryos are depicted with a 17% size reduction compared to *Drosophila* and *Ceratitis* embryos.

3.2 New genetic tools for improving SIT in *Ceratitis capitata*: embryonic lethality and sperm marking

In this part, the direct transfer of the *D. melanogaster*-derived embryonic lethality system (Horn and Wimmer, 2003) to *C. capitata* was tested. We found that an early embryonic promoter from *D. melanogaster* did not work in *C. capitata*. Therefore the isolation of endogenous homologs of *C. capitata* early embryonic promoters had to be done (3.3 and 3.4).
In addition, first experiments for generating a sperm marking system in *C. capitata* are presented. Further details are shown in 3.6.

Marc F. Schetelig, Francesca Scolari, Alfred M. Handler, Giuliano Gasperi and Ernst A. Wimmer

Author contributions to the practical work:

Marc F. Schetelig: All experiments for the embryonic lethality system and half the work for the sperm marking system

Francesca Scolari: Half the work for the sperm marking system

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New genetic tools for improving SIT in *Ceratitis capitata*: embryonic lethality and sperm marking

Marc F. Schetelig[1], Francesca Scolari[2], Alfred M. Handler[3], Giuliano Gasperi[2] & Ernst A. Wimmer[1]

[1] Department of Developmental Biology, Göttingen Center for Molecular Biosciences, Johann-Friedrich Blumenbach Institute of Zoology and Anthropology, Georg-August-University Göttingen, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany.

[2] Dipartimento di Biologia Animale, Università di Pavia, Piazza Botta 9, 27100 Pavia, Italy.

[3] USDA/ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida, USA.

Corresponding authors: mschete@gwdg.de; ewimmer@gwdg.de

Key words: cellularization, conditional embryonic lethality, insect transgenesis, sperm marker, *beta2-tubulin*.

Running head: Schetelig et al.: Embryonic lethality and sperm marking in *Ceratitis*

Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most devastating and economically important insects among 250 pest species belonging to the Tephritidae family (Khoo et al. 1991). A native of Sub-Saharan Africa, medfly has spread into the Mediterranean basin to parts of Central and South America, Hawaii and Australia in less than 200 years. Moreover, in the latter half of the last century, it was sporadically detected in different areas of the United States like California, Florida and Texas. The success of *C. capitata* infestation is partially dependant on its highly polyphagous nature (*C. capitata* attacks more than 250 different fruits, vegetables and nuts) and on the ability to adapt its multivoltine cycle to different temperate climates to overwinter as larvae in different crops.

Biological approaches to insect pest management offer alternatives to insecticidal control. The Sterile Insect Technique (SIT) is regarded as an ecologically safe method for area-wide control. SIT reduces the pest population by mass release of sterilized pest organisms (Knipling 1955). The SIT process involves mass rearing and release of individual flies rendered sterile through gamma or X-ray irradiation exposure. SIT has been proven in many areas to be effective against medfly. An example: Southern Mexico (MOSCAMED program), where the eradication of *C. capitata* was achieved over a region of 15.000 km^2 in 1982 (Hendrichs et al. 1983) and a barrier was created through Guatemala (Villasenor et al. 2000). For many pest species including medfly, SIT proved to be most effective by releasing only sterile males (Robinson 2002). However, the use of radiation for sterilizing insects does have some adverse effects on their competitiveness which in turn reduces the efficiency of the technique (Bushland 1971; Cayol et al. 1999). Recently a transgene-based embryonic sterility system was successfully established in *Drosophila melanogaster* (Meigen) (Horn and Wimmer 2003) and this system may provide an alternative to the use of radiation in area-wide integrated pest management programs involving the SIT.

Another problem in SIT programs is the monitoring: up to now in some SIT programs, mass reared pupae are sterilized and dusted with fluorescent powders which enable sterilized flies to be distinguished from wild flies when recaptured in traps in the release area. This monitoring system implies some disadvantages: the fluorescent dyes are expensive, dangerous for human health and error prone, because they can be transferred from sterilized marked flies to wild type ones (Hagler and Jackson 2001). Beside dyes, there have been genetic based approaches to improve the monitoring in various species. A dominant mutation has been isolated for *C. capitata*, which affects the third stripe on the abdomen (Niyazi et al. 2005). Field-cage studies have shown comparable sexual compatibility and mating competitiveness, including data on genetic sexing strains (GSS). Another system describes a transgenic sexing strain in *Anopheles stephensi* (Catteruccia et al. 2005). In this system the spermatogenesis specific *β2-tubulin* (*β2t*) promoter from *Anopheles gambiae* drives

enhanced green fluorescent protein (EGFP). This system has been proven under small-scale conditions as a good sexing strain in *Anopheles stephensi*.

Our aim is the development of a sperm marking system for *C. capitata*, which is based on the use of the *C. capitata* spermatogenesis-specific *β2t* promoter driving a fluorescent marker. After thorough strain evaluation and a test phase for fitness, accuracy and stability of the sexing procedure as well as the stability of these strains, they could be used for different purposes. A possible application might be the use as a transgenic sexing strain in combination with the ability for an easy monitoring in an operational SIT program. The system will also help in providing more detailed information on reproductive biology of *C. capitata.* The aim of the studies reported here was to establish and evaluate such embryonic lethality and sperm marking systems in *C. capitata*. Functional large-scale SIT activities, like those established for *C. capitata*, are ideal for comparing the effectiveness and usefulness of novel transgenic SIT approaches.

Material and Methods

In-situ hybridization. The RNA probes were made with a DIG-RNA-labeling Kit (Roche, Mannheim) and hybridizations were performed as described in Davis et al. (2001).

Plasmid. p*B*[*s1*-tTA; *PUb*-DsRed1] was constructed as previously described in Schetelig et al. (2007).

Results

1) Evaluation of a transgenic approach to sterilize flies with an embryonic lethal transgene combination in *C. capitata*

An approach to cause sterility was designed without interfering with spermatogenesis to maintain males and their sperm as competitive as possible. We followed a strategy based on the expression of a lethal factor under the control of a promoter that is active at early blastoderm stages. When the male is homozygous for the combination of the necessary gene constructs, each fertilization event will lead to embryonic lethality (Horn and Wimmer 2003). The advantage of this system lies in the proposed high competitiveness of such males, since their reproductive organs will not be affected and matings actually lead to sperm transfer. However, it is very important that the promoter is active only in early stages of development. Then the lethal phase can be overcome while developing under permissive conditions in the rearing facilities, whereas after release non-permissive conditions will not affect the males themselves but only their progeny (Wimmer 2005). The system employs the ectopic expression of a hyperactive proapoptotic gene that causes embryo-specific lethality when driven by the tetracycline-controlled transactivator tTA under the regulation of a cellularization gene

enhancer/promoter. The system has been tested successfully in *Drosophila melanogaster* (Horn and Wimmer 2003). We want to transfer this binary expression system to *C. capitata* to evaluate it in comparison with the effective conventional SIT using radiation-induced sterility.

We first tried the direct transfer of the Drosophila-derived system to *C. capitata*. Therefore we injected the driver construct p*B*[*s1*-tTA; *PUb*-DsRed1], which contains the tTA gene under the control of the *Drosophila melanogaster serendipity α (sry α)* promoter region (Schetelig et al. 2007), together with the p*hsp*-p*Bac* (Handler and Harrell 1999) into the germline of *C. capitata*. We got four independent transgenic lines. These lines where tested for tTA expression by whole mount *in-situ* hybridizations with a RNA probe to tTA. None of the four transgenic lines expressed the *tTA*. Representative three of the four lines are shown in Figure 1.

Thus, the cellularization specific *sry α* promoter from *Drosophila* seems not to be functional in *C. capitata*. In order to get functional promoters for use in our system, we searched for endogenous promoters of *C. capitata*. To obtain *C. capitata* genes and their promoters, which lead to specific expression at the blastoderm stage, we first carried out PCR-based cDNA subtractions of different embryonic stages and identified several cellularization-specific genes (Schetelig et al. 2007). After that we isolated the corresponding enhancers/promoters by inverse PCR (iPCR) and subsequently brought the tTA independently under the control of each enhancer/promoter region. We injected the construct carrying the cellularization specific promoter of *sub1_68* from *C. capitata* into the germline of *C. capitata* and got transgenics with cellularization specific expression of tTA (Figure 2). However, the expression as detected by whole mount *in-situ* hybridizations was relatively weak.

2) Development of a transgenic sperm marking system for *Ceratitis capitata*

To develop a sperm marking system for the fruit fly *C. capitata*, we isolated the spermatogenesis specific gene *β2t* from *C. capitata* by degenerate primer PCR. By rapid amplification of cDNA ends (RACE), we amplified the complete *β2t* and isolated the upstream region by iPCR. The observed upstream region of *β2t* was fused to the fluorescent marker turboGFP (tGFP). *β2t*-tGFP was then inserted in a *piggyBac* (p*B*) transposon vector carrying a red (DsRed) fluorescent marker driven by a polyubiquitin promoter (*PUb*): *β2t*-tGFP in p*B*[*PUb*-DsRed1]. The advantage of this transposon vector is that it carries a characterized fluorescent marker driven by the ubiquitiously expressed polyubiquitin promoter from *Drosophila melanogaster* (Handler and Harrell 2001). The construct p*B*[*β2*-tGFP; *PUb*-DsRed1] was injected with a transposase source into the posterior end of *C. capitata* embryos to cause germline transformation. Flies from this transformation have a red body and males have green fluorescent testes (Figure 3). For sperm use and remating analyses different fluorescent marked sperms would be helpful. Therefore we are developing also a system carrying a green body and a red testes marker.

Discussion

1) Evaluation of a transgenic approach to sterilize flies with an embryonic lethal transgene combination in *Ceratitis capitata*

A direct transfer of the driver construct carrying the cellularization specific *D. melanogaster sry α* promoter to *C. capitata* showed that this promoter is not functional in *C. capitata*. Thus, we can assume that the complex interaction between enhancers and promoters of stage-specifically expressed genes (Blackwood and Kadonaga 1998) is different between *D. melanogaster* and *C. capitata*. A *Drosophila melanogaster* promoter might not act as an adequate alternative to an endogenous *C. capitata* promoter to enable strong expression rates.

For a highly specific embryonic lethality system we need promoters mediating strong expression specific to early embryonic stages. Therefore we isolated several cellularization genes from *C. capitata* (Schetelig et al. 2007) and their promoters. We could show that the *Ceratitis* cellularization specific promoter from *sub1_68* fused to tTA expressed tTA specifically at cellularization stages (Figure 2). So this promoter might be sufficient for using it for our embryonic lethality system. But the staining time for the color reaction of 24h for the depicted whole mount *in-situ* hybridizations was not in an estimated average time window for *in-situ* hybridizations on *C. capitata* with RNA probes (average of staining time to the color reaction is completed in *C. capitata* embryos is between 0.5 and 3h). So we suppose that the specific, albeit low expression of this construct will probably not be sufficient for generating a successful and safe lethality system. To better promote the tTA expression we wish to isolate varying long versions of the *sub1_68* upstream region which might include more regulating elements. We will also isolate and test upstream regions from other *C. capitata* cellularization specific genes.

2) Development of a transgenic sperm marking system for *Ceratitis capitata*

After thorough strain evaluation and test phase for fitness, accuracy and stability, the developed sperm marked strains are a first step for improving the following objectives:

- Reproductive biology in *Ceratitis capitata*. Thus far little is known about the mechanisms of remating behavior and the consequential sperm use. With the sperm marking system it should be possible to widen our the knowledge of the reproductive biology of the medfly. The obtained results will also be important to further improve strategies of SIT programs.
- Monitoring of an area wide SIT program. Because *β2t* is expressed in the testes, released males from these strains could be distinguished from wildtype males in the monitoring process during SIT. Together with the information on trapped females in the field and dissection of their sperm storage organs, this would allow for a better evaluation of the efficiency of SIT programs.

- In principle these strains can also be used as sexing lines that could be sorted automatically for males and females during larval development (presuming gonadal fluorescence is detectable). However, since *Ceratitis capitata* has already an effective sexing strain, this might be more important for the development of SIT in other Tephritids.

Acknowledgements

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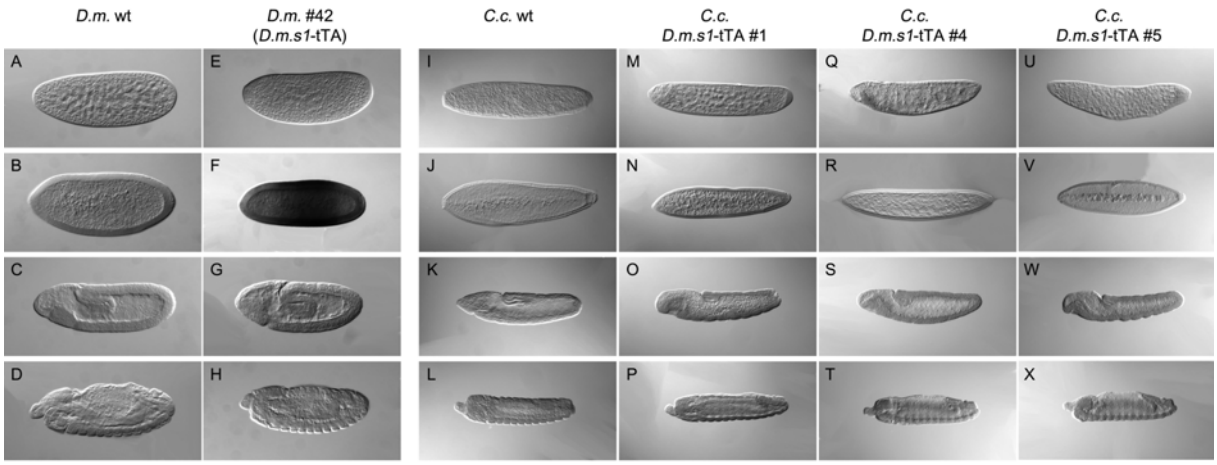

Figure 1. Cellularization specific promoter of *Drosophila* is apparently nonfunctional in *C. capitata*

Shown are whole mount *in-situ* hybridizations using a tTA RNA probe. The embryogenesis of each strain (columns) is pictured with four embryonic stages: first row = blastoderm; second row = cellularization; third row = germ band elongation; fourth row = germ band retraction. *D.m.* wildtype (A-D): no expression of tTA at all stages. *D.m.* #42 (Horn and Wimmer 2003) (E-H): tTA is cellularization specifically expressed in this strain, which carries the *D.m.s1*-tTA. *C.c.* wildtype (I-L): no expression of tTA at all stages. *C.c. D.m.s1*-tTA #1 (M-P), *C.c. D.m.s1*-tTA #4 (Q-T) and *C.c. D.m.s1*-tTA #5 (U-X): no expression of tTA at all stages.

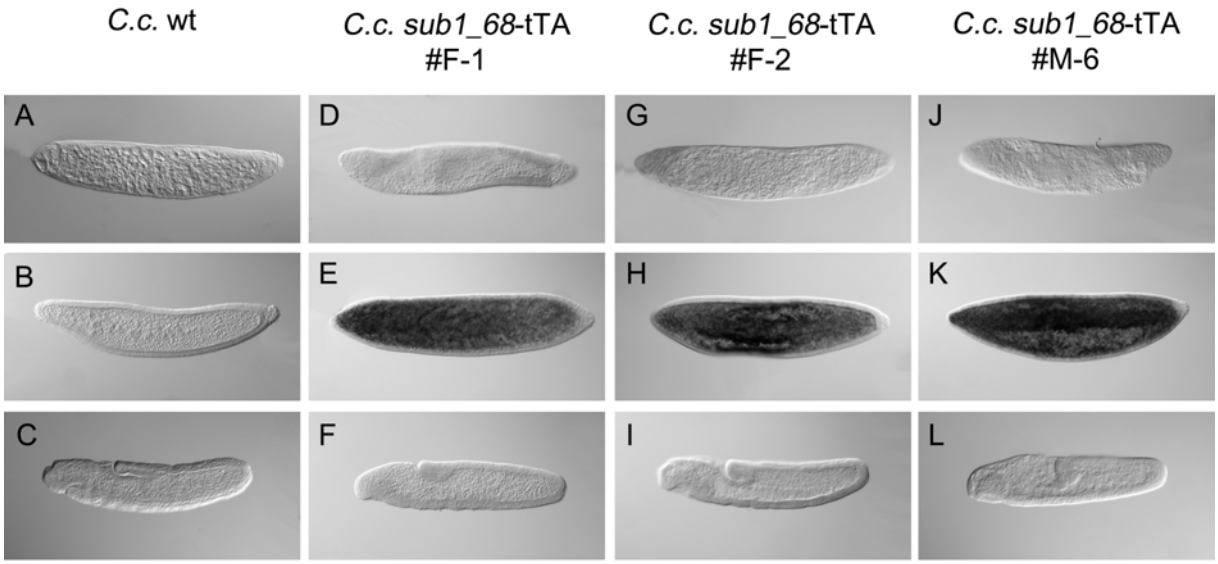

Figure 2. Endogenous promoter of a cellularization specific gene of *C. capitata* causes weak tTA expression

Shown are whole mount *in-situ* hybridizations using a tTA RNA probe. The embryogenesis of each strain (columns) is pictured with three embryonic stages: first row = blastoderm; second = cellularization; third = germ band elongation. *C.c.* wildtype control (A-C): no expression of tTA at all stages. *C.c. sub1_68*-tTA #F-1 (D-F), *C.c. sub1_68*-tTA #F-2 (G-I) and *C.c. sub1_68*-tTA #M-6 (J-L): cellularization specific expression of tTA driven by the *C.c. sub1_68* promoter.

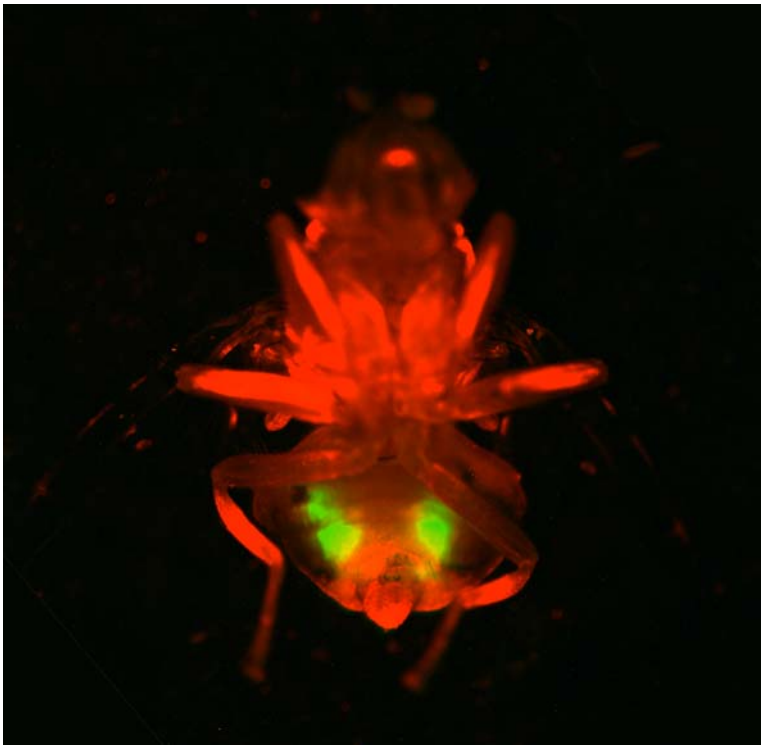

Figure 3. Transgenic marked *C. capitata* male carrying two different types of molecular markers

The male has a red fluorescent body marker (*PUb*-DsRed) and a testes-specific green fluorescent marker (*β2t*-tGFP).

3.3 Development of an embryonic lethality system for SIT in *Ceratitis capitata*

In this part the first isolation of early embryonically expressed medfly genes is described. First, I tried to isolate the medfly cellularization genes *nullo* and *serendipity α* by degenerate primer PCR based on sequence similarities to drosophilid genes. Because I could not obtain the cellularization genes with this method, I characterized the early embryogenesis of *C. capitata* to carry out stage-specific screening. I found that the cellularization in *C. capitata* takes place later and for a longer period compared to *D. melanogaster*. With this knowledge, a stage specific screen for early embryonic genes was performed by using the cDNA subtraction method. I isolated six genes, which had cellularization specific expression patterns. The use of their promoters turned out to be a key point in generating a successful embryonic lethality system in *C. capitata* (see 3.4).

Schetelig, M. F., Horn, C., Handler, A. M. and Wimmer, E. A. (2007).

Author contributions to the practical work:

Schetelig, M. F.:	All experiments, besides*
Horn, C.:	*Development of the transgene-based sterility system in *D. melanogaster*
Handler, A. M.:	*Generation of four transgenic medfly lines carrying the construct p*B*[*s1*-tTA; *PUb*-DsRed1]

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Development of an Embryonic Lethality System in Mediterranean fruit fly *Ceratitis capitata*

M. F. SCHETELIG[1], C. HORN[2], A. M. HANDLER[3] and E. A. WIMMER[1]

[1]*Department of Developmental Biology, Johann-Friedrich Blumenbach Institute of Zoology and Anthropology, Georg-August-University Göttingen, Justus-von-Liebig-Weg-11, 37077 Göttingen, Germany*
[2]*Structural and Computational Biology Unit, EMBL Heidelberg, Meyerhofstraße 1, 69117 Heidelberg, Germany*
[3]*USDA/ARS, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL 32608, Florida, USA*

ABSTRACT The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) is one of the world´s most destructive insect pests, costing farmers billions of dollars annually. Improved biological strategies are needed to increase the efficacy of area-wide integrated pest management (AW-IPM) programmes. Transgenic methodology could enhance and widen the applicability of the sterile insect technique (SIT) as a component of AW-IPM programmes and a transgenic approach to sterilize insects with an embryonic lethal transgene combination instead of conventional radiation was successfully tested in *Drosophila melanogaster* Meigen. This system is currently being transferred to *C. capitata*, in order to test its feasibility in this species and compare its effectiveness to radiation sterilization. Therefore two strategies are being followed: (1) direct transfer of the constructs used in *D. melanogaster* and assessment of their functionality in *C. capitata*, and (2) isolation of genes active during early embryonic development of *C. capitata* for use in an embryonic lethality system with endogenous components. If proven functional and effective in *C. capitata*, such a system might be transferable to other insect pests.

KEY WORDS *Ceratitis capitata*, cellularization, cDNA-subtraction, conditional embryonic lethality, sterile insect technique (SIT), insect transgenesis

1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) is one of the worlds' most important pests of fruits and vegetables, attacking more than 260 different fruits, vegetables and nuts. The direct damage caused by feeding larvae and the quarantine status of this insect have major impacts on many agricultural economies. Biological approaches to insect pest management offer alternatives to insecticidal control. The sterile insect technique (SIT) is a powerful component of area-wide integrated pest management (AW-IPM) programmes to suppress or eliminate populations of economically important pest species by the mass-release of radiation-sterilized insects (Knipling 1955). However, the use of radiation for sterilizing insects does have some adverse effects on their competitiveness which in turn reduces the efficiency of the technique (Cayol et al. 1999, Calkins and Parker 2005).

Recently a transgene-based embryonic sterility system was successfully established in *Drosophila melanogaster* Meigen (Horn

and Wimmer 2003) and this system may provide an alternative to the use of radiation in AW-IPM programmes integrating the SIT. The aim of the studies reported here was to establish and evaluate such a system in *C. capitata*.

2. Transgene-Based Sterility System in *Drosophila melanogaster*

A novel transgenic approach was developed to induce sterility without interfering with gametogenesis or with other larval and adult stages of the insect life cycle. Sterility is based on the transmission of a transgene combination that causes dominant embryo-specific lethality in subsequent progeny. This dominant lethality is suppressible by additives in the larval diet, thereby enabling rearing of such strains. This should allow the generation of competitive sterile insects that can transfer competitive sperm (Horn and Wimmer 2003), carrying the transgene, to wild females. The embryos produced by the females will carry the dominant transgene and, in the absence of the additives, the embryos will die. For the effector gene causing organismal lethality, a hyperactive allele of the pro-apoptotic gene *head involution defective* (*hid*) was chosen, which induces cell death when expressed ectopically (Grether et al. 1995). To avoid down regulation of HID by developmental signalling pathways, the phosphoacceptor-site mutant allele hid^{Ala5} (Bergmann et al. 1998) was used. To limit the effect of the transgenes to the embryonic stage, enhancer-promoters of genes that are expressed at high levels but are specific to the cellularization stage were used. In *D. melanogaster* the genes *serendipity α (sry α)* and *nullo* encode structural components of the microfilament network that are specifically required for blastoderm cellularization (Ibnsouda et al. 1993, Postner and Wieschaus 1994). To establish conditional embryonic lethality, a suppressible binary expression system based on the tetracycline controlled transactivator tTA (Gossen and Bujard 1992) was employed. By adding tetracycline to the larval diet the transgene activity can be suppressed. In *D. melanogaster*, hid^{Ala5} specifically causes embryonic lethality when driven by tTA under the control of the enhancer-promoter from a cellularization gene, and can be suppressed by tetracycline provided maternally to the egg (Horn and Wimmer 2003). Due to the inhibition of the tTA-DNA binding by tetracycline, the tTA protein functions as a switch to discriminate restrictive from permissive conditions. Under restrictive conditions (without tetracycline) 99.9% of *D. melanogaster* embryos that inherited one copy of the transgene combination were killed. Under permissive conditions (with tetracycline), lethality was suppressed which allowed the continuous generation of large numbers of transgenic insects (Fig. 1). Strains homozygous for the transgene combination can be propagated on tetracycline-containing food. Males from these *D. melanogaster* strains are competitive in laboratory mating assays and transmit the transgene combination, which causes dominant embryonic lethality in offspring. Thus the transgene-based suppressible embryo-specific lethality system may enable competitive sterile insects to be produced without irradiation and is therefore of interest for improving conventional SIT and widening its applicability.

3. Transfer of the Transgene Embryonic Lethality System to *Ceratitis capitata*

3.1. Direct Transfer of the Drosophila*-Used Transgenes to* Ceratitis capitata

For fast and easy transfer of the embryo-specific lethality system from the model organism to the pest species, direct use of the *D. melanogaster* transformation constructs in *C. capitata* was pursued (Horn and Wimmer 2003). This involved taking the driver construct pBac{3xP3-EYFP;>>s1-tTA>>} (Horn and Wimmer 2003) and digesting it with *Bgl*II. The fragment, which contains the *tTA* gene under control of the -276:+45 *sry α* promoter region, was inserted into the *Bgl*II site of the transformation vector pB[PUb-

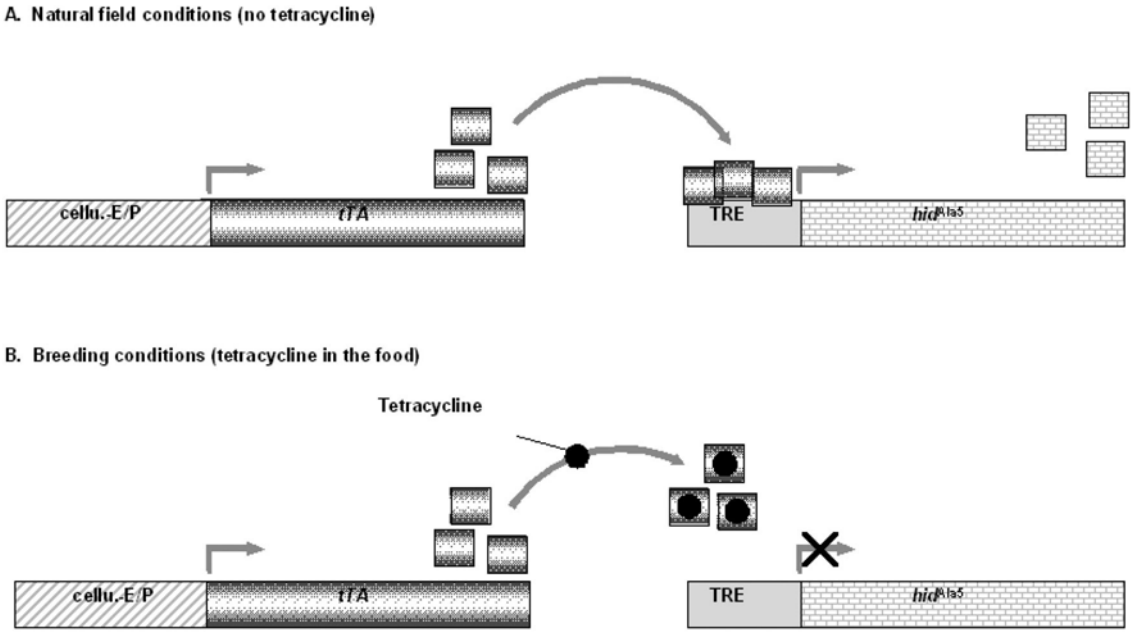

Figure 1. *Binary expression system for conditional embryonic lethality. Enhancer-promoters of the cellularization genes (cellu.-E/P)* sry α *or* nullo *were selected, which mediate gene expression exclusively during early embryogenesis. The tetracycline controlled transactivator (tTA) is based on a bacterial-viral fusion protein and mediates gene expression by binding to a* tTA-response element (TRE)*. The main advantage of this system is that targeted gene expression can be controlled by the food supplement tetracycline. (a) Under natural field conditions there is no tetracycline and the tTA proteins bind to the* TRE *leading to the expression of* hid^{Ala5}*, which causes lethality. Induction of lethality is limited to the early cellularization stage of embryogenesis because the genes* sry α *and* nullo*, are exclusively expressed at the cellularization stage. (b) Under laboratory rearing conditions the larval diet contains tetracycline, which binds to tTA. Tetracycline-bound tTA cannot bind to the* TRE *thereby suppressing* hid^{Ala5} *expression and allowing all progeny to survive.*

DsRed1] (Handler and Harrell 2001). For germ-line transformation this construct was injected together with a helper plasmid into the posterior of early *C. capitata* embryos, resulting in four transgenic lines. These are currently being analysed for transgene-mediated *tTA* expression.

The results will determine: (1) whether the complex interaction between enhancers and promoters of stage-specifically expressed genes (Blackwood and Kadonaga 1998) is the same or different between *D. melanogaster* and *C. capitata,* and (2) whether a *D. melanogaster* promoter can act as an adequate alternative to an endogenous *C. capitata* promoter to enable high expression rates to be obtained.

3.2. Embryonic Cellularization Genes from Ceratitis capitata

3.2.1. Searching for sry α and nullo *by Degenerate Polymerase Chain Reaction (PCR)*

According to fossil records, the phylogenetic distance between *Drosophila* spp. and *Ceratitis* spp. can be estimated to be around 100 million years (Naumann 1994). Since the cellularization genes *sry α* and *nullo* are fast evolving even within the Drosophilidae (Ibnsouda et al. 1993, Hunter et al. 2002), it might be challenging for the *D. melanogaster* constructs to function in a more distantly related species such as *C. capitata*. Therefore, we started to isolate cellularization genes *sry α* and *nullo* in *C. capitata*.

To obtain specific DNA sequences of *C. capitata* homologues of the genes *nullo* and *sry α*, mRNA from 0-48 hour embryo collections was isolated and translated into double-stranded cDNA for later PCRs. Degenerate primers were designed on the basis of amino acid sequence comparisons between known drosophilid *nullo* and *sry α* proteins (Ibnsouda et al. 1998, Hunter et al. 2002) under the following conditions: primer length between 18-29 base pairs and a maximum of 64 permutations. Using the cDNA collection and the degenerate primers, gradient PCRs were carried out (annealing temperature: gradient from 39°C to 50°C) with all suitable primer combinations. If possible, nested PCRs were performed after the primary gradient PCRs for a more selective amplification and a reduction of background. As a control, gradient PCRs were carried out with only one of the degenerate primers to check whether these already lead to non-specific amplifications. The DNA fragments of possibly interesting bands were cut out of an agarose gel, purified (QiaEX II Gel Extraction Kit, Qiagen, Hilden), ligated into the vector pCRII (TA Cloning Kit Dual Promoter (pCRII), Invitrogen) and transformed. The DNA clones were sequenced and analysed by "basic local alignment search tool" (BLAST) algorithms (Altschul et al. 1997) as well as *in situ* hybridizations to whole mount *C. capitata* embryos.

Unfortunately none of the BLAST hits matched the *nullo* or *sry α* genes from drosophilids. Also none of the *in situ* hybridizations with probes from sequences with no BLAST hits gave expression patterns comparable to *D. melanogaster nullo* or *sry α*. Thus the cellularization-specific genes could not be obtained by this degenerate PCR approach based on sequence similarities to drosophilid genes. One reason for this might be the fast evolution of developmental genes in drosophilids (Schmid and Tautz 1997).

3.2.2. Cellularization in Ceratitis capitata

Because *nullo* and *sry α* homologues from *C. capitata* could not be isolated by PCR with degenerate primers, blastoderm-specifically expressed genes were isolated in an independent experiment. For this purpose, we first determined the time window of cellularization in *C. capitata* and this knowledge was used to select differentially expressed genes by cDNA subtractions (3.2.3.).

To determine the time window of cellularization, embryos were fixed at one hour intervals after oviposition followed by immunofluorescence staining of cell membranes and nuclei. For comparison, the same staining was done on *D. melanogaster* whose embryonic development lasts 22 hours at 25°C and whose cellularization takes place between 2 hours 10 minutes and 2 hours 40 minutes after oviposition. In contrast to *D. melanogaster*, embryonic development in *C. capitata* takes 48 hours and cellularization takes place later and for a longer period from nine to 12 hours after oviposition (Fig. 2). In *C. capitata*, the typical elongation of the nuclei could not be observed during the slow phase of cellularization as described for *D. melanogaster* (Lecuit and Wieschaus 2000).

3.2.3. Enrichment of Cellularization- Specific Gene Transcripts by cDNA-Subtraction

In *D. melanogaster* cellularization genes are highly and exclusively expressed during the superficial cleavage of insect embryos (Postner and Wieschaus 1994, Lecuit and Wieschaus 2002). Thus isolating one or more of these genes and particularly their promoters in *C. capitata* would allow a *C. capitata*-specific embryonic lethality system to be generated. With knowledge of the cellularization time window (3.2.2.) a stage-specific screening was performed. Since strongly expressed genes, which exist during all stages of embryogenesis, would prevent a successful and effective cDNA screen for cellularization-specific genes, a cDNA subtraction approach was used (Diatchenko et al. 1996) for the selective isolation of genes, which are specifically expressed during *C. capitata* cellularization.

Using the cDNA transcripts isolated from the cellularization stage those cDNA transcripts, which are also present in other embry-

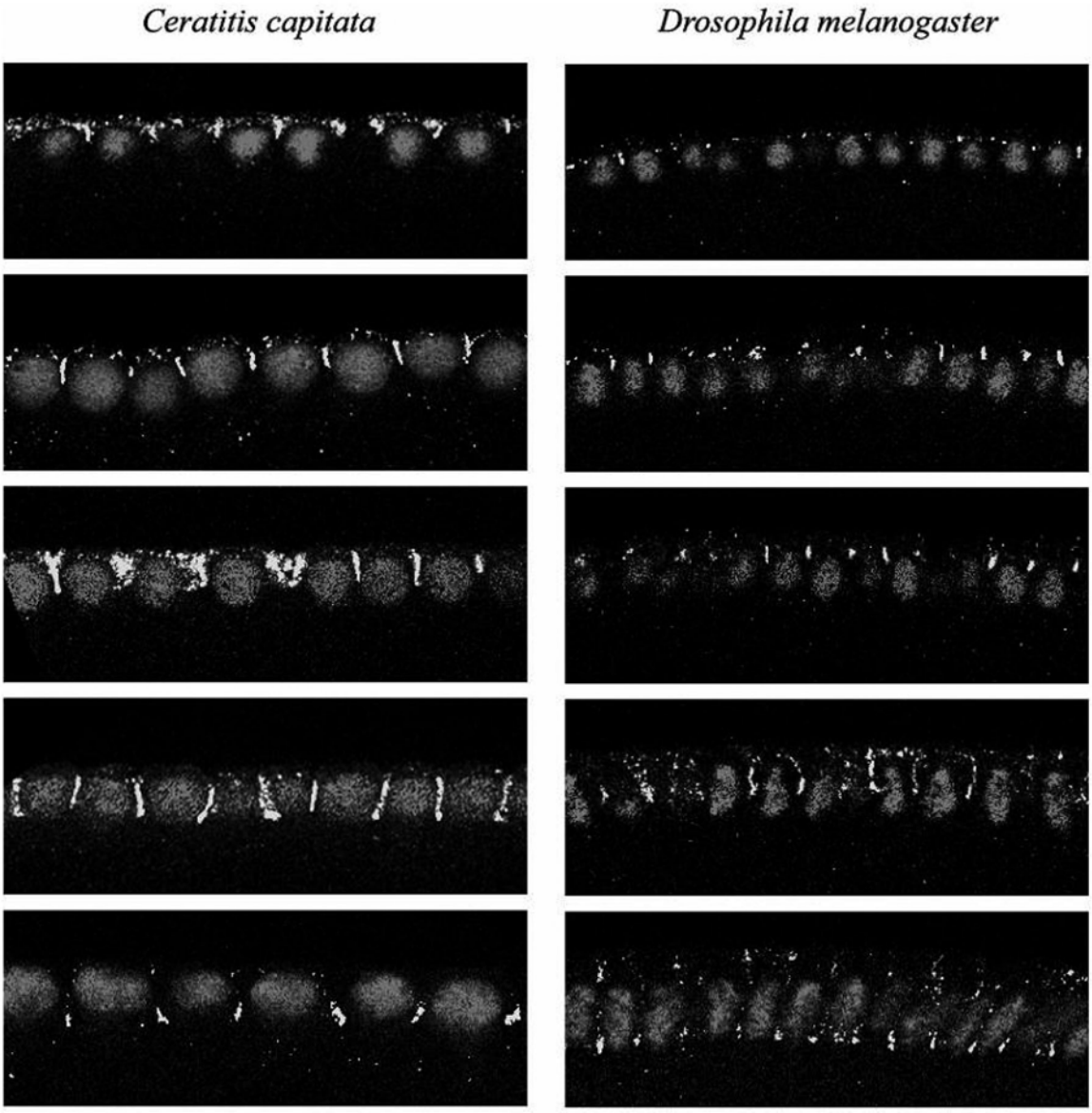

Figure 2. *Superficial cleavage during insect development. Comparison of the cellularization of (left side)* Ceratitis capitata *and (right side)* Drosophila melanogaster *embryos. The immunofluorescence staining with primary armadillo antibody and secondary Alexa488-marked antibody shows the invagination of the cell membrane (bright white stripes between the large gray nuclei). Nuclei are stained with propidiumiodide (large gray balls). In* C. capitata*, cellularization takes places between nine hours (upper, left panel) and 12 hours (lower, left panel) after oviposition. In* D. melanogaster*, cellularization takes places between two hours and ten minutes (upper, right panel) and two hours and 40 minutes (lower, right panel) after oviposition. Panels between represent intermediate stages of cellularization in chronological (vertical) order.*

onic developmental stages, were subtracted. Two subtractions were carried out: (1) a [0-6 hours + 15-21 hours] double-stranded cDNA collection from a double-stranded cDNA collection of cellularization stages (9-12 hours), and (2) a [0-6 hours + 15-48 hours] double-stranded cDNA collection from a double-stranded cDNA collection of a widened "cellularization" time window (7.30-12.30 hours).

The second subtraction was performed because only 4% of the isolated genes in the first subtraction were identified as cellulariza-

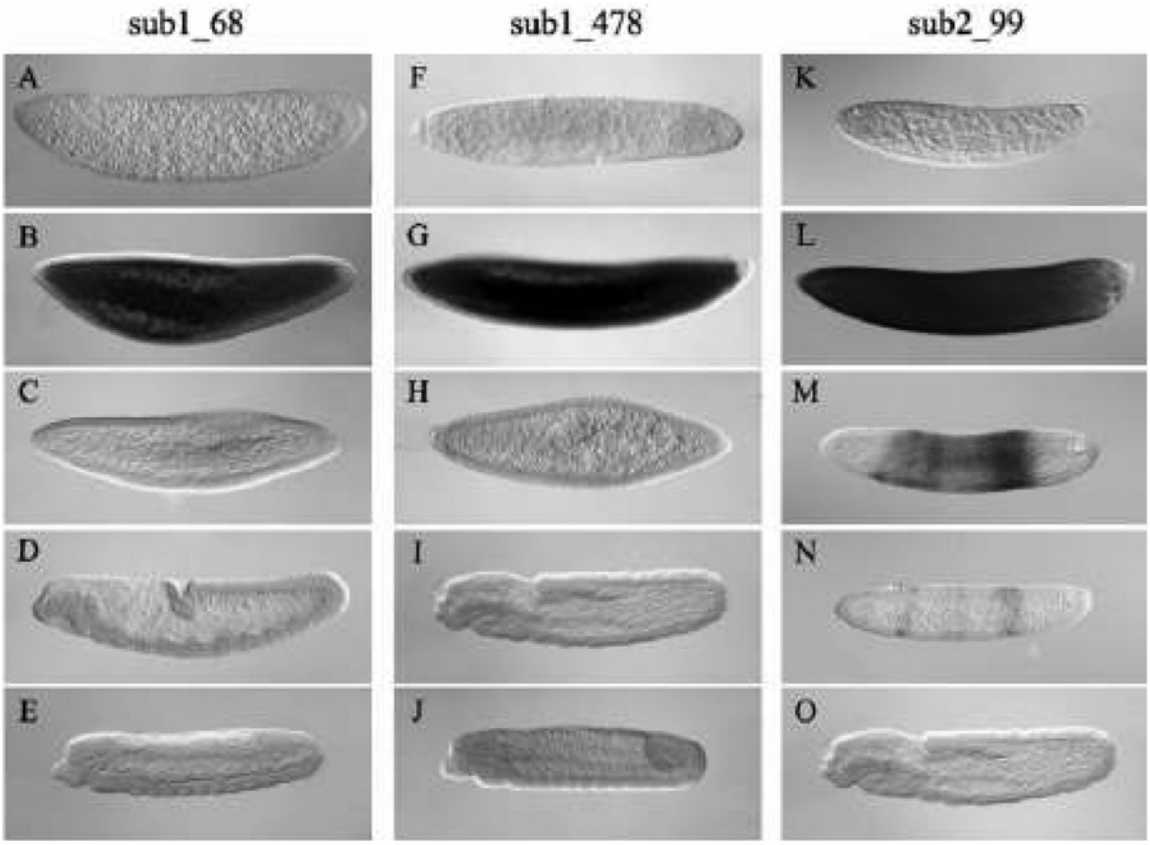

Figure 3. *Cellularization-specific gene expression patterns 1.* Ceratitis capitata *gene sequences* ***sub1_68*** *and* ***sub1_478.*** *(a) and (f) preblastodermal embryo without expression, (b) and (g) strong expression at the onset of cellularization, (c) and (h) very weak expression at the end of the cellularization, (d) and (i) no expression during gastrulation, (e) and (j) no expression during after germ band retraction.* ***Sub2_99*** *(k) preblastodermal embryo without expression, (l) strong expression at the onset of cellularization, (m) strong expression restricted to the centre of the embryo and lacking at the anterior and posterior pole during the slow phase of cellularization, (n) weak expression reduced to three stripes in the centre of the embryo at the fast phase of cellularization, and (o) no expression during and after germ band elongation.*

tion specific by *in situ* hybridizations and BLAST searches. In addition many housekeeping genes had been amplified. The second subtraction conditions were improved by using a widened cellularization time window to make sure that cellularization sequences, produced at earlier time points and also responsible for cellularization, could be identified. Furthermore, the subtracted cDNA pool was expanded to 48 hours to improve the exclusion of non-differentially expressed genes. This increased the efficiency to ~12%. PCR products from these subtractions were agarose gel purified, ligated and transformed into the vector pCRII (for details see 3.2.1). Transformants were pre-selected by restriction enzyme digest patterns and their plasmids then isolated and sequenced. The DNA clones obtained were analysed by *in situ* hybridizations to whole mount *C. capitata* embryos.

From 720 transformants (subtraction 1 (sub1): 550; subtraction 2 (sub2): 170), putative identical clones were identified by enzyme restrictions and 106 probably different clones sequenced (sub1: 45; sub2: 61). Six of the 106 clones were expressed exclusively during cellularization of *C. capitata* (sub1: two; sub2: four; Figs. 3 and 4). Additional three clones were highly expressed during cellularization, but their expression was not

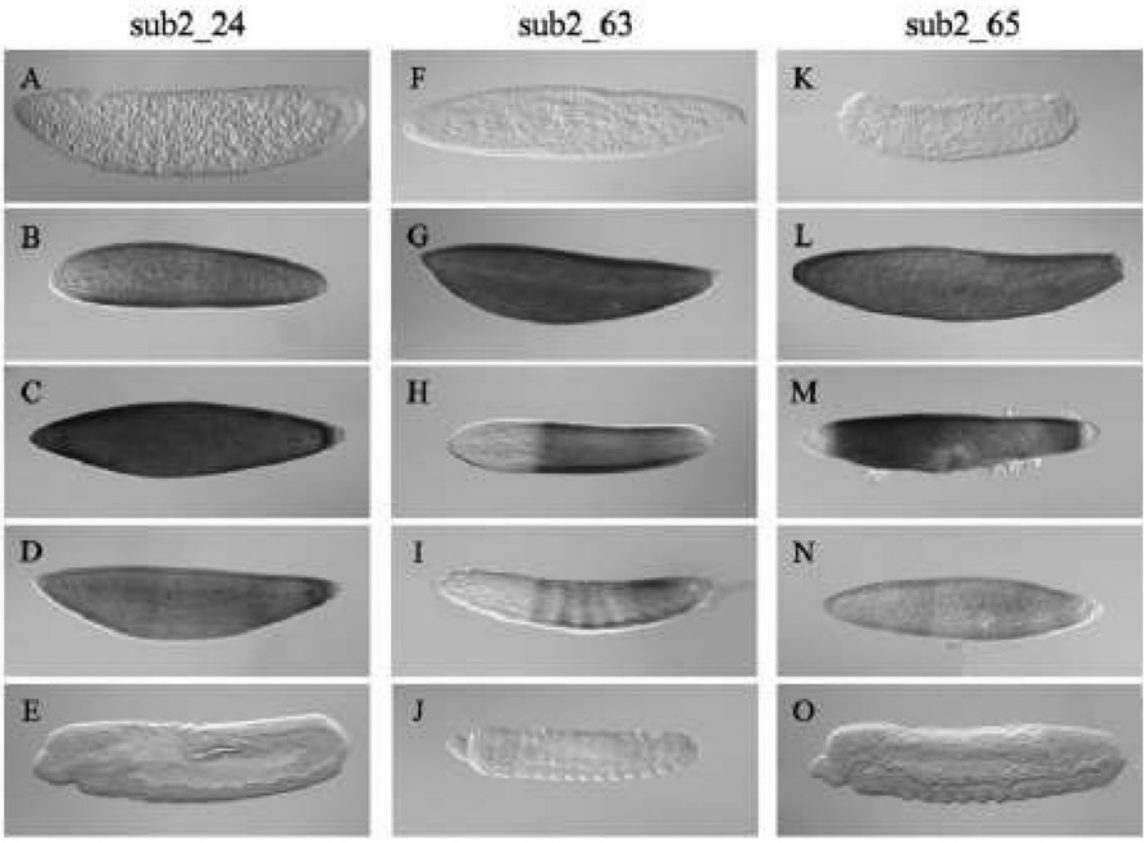

Figure 4. *Cellularization-specific gene expression patterns 2.* Ceratitis capitata *gene sequences* ***sub2_24****. (a) preblastodermal embryo without expression, (b) strong expression at the onset of cellularization, (c) very strong expression during the slow phase of cellularization, (d) reduced expression during the fast phase of cellularization, (e) no expression during germ band retraction.* ***sub2_63*** *(f) preblastodermal embryo without expression, (g) strong expression at the onset of cellularization, (h) strong expression exclusive of the posterior and anterior pole in the slow phase of cellularization, (i) weak expression ending up in stripes exclusive of the posterior and anterior pole in the fast phase of cellularization, (j) no expression after germ band retraction.* ***sub2_65 (k)*** *preblastodermal embryo without expression, (l) strong expression at the onset of cellularization in the whole embryo, (m) very strong expression exclusive of the anterior and posterior pole during the slow phase of cellularization, (n) weak expression reduced to broad stripes at the fast phase of cellularization, and (o) no expression after germ band retraction.*

restricted to this stage (data not shown).

4. Conclusions

A transgene-based embryonic lethality system established in *D. melanogaster* is being evaluated following injection of the driver construct of the binary expression system into *C. capitata* embryos. It will be interesting to determine whether the *sry α* promoter from *D. melanogaster* is also active in *C. capitata* as well as to what extent and in which stages it leads to expression.

To search for cellularization-specific genes in *C. capitata* by cDNA subtraction, the cellularization time window of *C. capitata* embryogenesis was first determined. Six cellularization-specifically expressed candidate genes were isolated by the cDNA subtraction screen. Current work involves searching for the promoter/enhancer regions of these genes by inverse PCR of genomic DNA for use in a

C. capitata specific transgene-based embryonic lethality system.

Once the promoters/enhancers are available, a transgenic sterility system for *C. capitata* will be constructed and its fitness and competitiveness compared to flies sterilized by radiation.

5. Methods

Secondary antibodies (Jackson Immunoresearch) were obtained commercially. The anti-Armadillo antibody (mAb N2 7A1) (Peifer et al. 1994) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Antibody stainings were performed as described by MacDonald and Struhl (1986). For the cDNA subtraction the Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, Heidelberg) was used. The RNA probes for *in situ* hybridization were made with DIG-RNA-labelling Kit (Roche, Mannheim) and hybridizations were performed as described in Davis et al. (2001).

6. Acknowledgements

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3.4 Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae)

In this part the development of the first transgenic embryonic lethality system for medfly using an early embryonic lethal transgene combination is reported. The system is generated using the binary expression system Tet-Off, which is suppressible by tetracycline and consists of a driver construct, which carries an embryonically active promoter from *C. capitata* driving the *tetracycline-controlled transactivator* (*tTA*), and an effector construct, which carries a tTA responsive element (TRE), a basal promoter and a proapoptotic gene (hid^{Ala5}). The system can be activated by the absence of tetracycline in the food. Then the embryonically expressed tTA can bind to TRE, which leads to the expression of the lethal factor (see also 3.3, Figure 1, p. 54). The developmental stage and level of lethality in such transgenic lines was highly dependent on the newly isolated endogenous promoter/enhancer elements of cellularization-specifically expressed genes from medfly (see 3.3), which were used to establish the necessary driver constructs of the system. We show that these elements act differently in expression strength and their ability to activate lethal effector constructs. 60 different combinations of driver and effector construct integrations were tested with one line showing complete embryonic lethality. We demonstrate that the efficiency of the killing system is dependent on the integration sites of driver and effector constructs. The line showing complete embryonic lethality was highly competitive to wildtype medfly in laboratory and field cage tests. This embryonic lethal line could become an alternative to medfly strains, which have to be sterilized by irradiation, so that the complete step of irradiation during SIT programs could be skipped in the future.

Marc F. Schetelig, Carlos Caceres, Antigone Zacharopoulou, Gerald Franz and Ernst A. Wimmer

Author contributions to the practical work:

Marc F. Schetelig:	All experiments, besides*
Carlos Caceres:	*Field cage competition tests
Antigone Zacharopoulou:	**In-situ* hybridizations on spread chromosomes

STATUS: SUBMITTED. For sequences see appendix 6.2.

PATENT FILED: Application No. 61/038,210; Title: Developmental stage-specific lethality system for insect population control

Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae)

Marc F. Schetelig[*§], Carlos Caceres[†], Antigone Zacharopoulou[†], Gerald Franz[†] and Ernst A. Wimmer[*§]

* Department of Developmental Biology, Göttingen Center for Molecular Biosciences, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, Georg-August-University Göttingen, GZMB, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany.

[†] International Atomic Energy Agency, FAO/IAEA Agriculture and Biotechnology Laboratory Entomology Unit, A2444 Seibersdorf, Austria.

§ To whom correspondence should be addressed. e-mail: mschete@gwdg.de; ewimmer@gwdg.de.

Author contributions: MFS and EAW designed research; MFS, CC and AZ performed research; MFS, CC, GF and EAW analyzed data; and MFS and EAW wrote the paper.

Accession numbers: in preparation

Abstract

The sterile insect technique (SIT) is an environment-friendly method used in area-wide pest management of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann). Ionizing radiation used to generate reproductive sterility in the mass-reared populations before release leads to reduction of competitiveness. Here, we present a first alternative reproductive sterility system for medfly based on transgenic embryonic lethality. This system is dependent on newly isolated medfly promoter/enhancer elements of cellularization-specifically expressed genes. These elements act differently in expression strength and their ability to drive lethal effector gene activation. Moreover, position effects strongly influence the efficiency of the system. Out of sixty combinations of driver and effector construct integrations, several lines resulted in larval and pupal lethality with one line showing complete embryonic lethality. This line was highly competitive to wildtype medfly in laboratory and field cage tests and thus can be used to improve the efficacy of operational medfly SIT programs.

Keywords: medfly, pest management, sterile insect technique, transgenesis, Tet-Off

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae), is one of the most devastating and economically important insect pests (White & Elson-Harris, 1992). An effective biological and environmental-friendly control of this pest is the sterile insect technique (SIT) (Enkerlin, 2005). The SIT reduces a pest population by mass release of reproductively sterile male insects into a wild type (WT) population of the same species. This leads to the decrease of progeny by competition of sterilized males with WT males for WT females (Knipling, 1955). Thus, the sterilization of the pest species in SIT programs is of major importance and is commonly induced by radiation. However, the sterility and competitiveness are indirectly correlated (Parker & Mehta, 2007). In some programs therefore lower doses of radiation are used to generate lines, which are more competitive even though only partially sterile. In preventional release programs, completely sterile flies are released into pest free areas to avoid the establishment of invasive fruit flies and to control the constant problem of re-infestations (Hendrichs *et al*, 2002). These programs have to use 100% sterile flies to avoid a novel introduction of insect pests. However, the competitiveness of such flies is reduced due to the high dose of radiation required for complete lethality. A first approach to cause reproductive sterility by transgene-based embryonic lethality without the need of radiation has been successfully shown in the non-pest insect *Drosophila melanogaster* (Horn & Wimmer, 2003). The system is based on the transmission of a transgene combination that causes embryo-specific lethality in the progeny. To limit the effect of the transgenes to the embryonic stage, promoter/enhancers (P/Es) from cellularization-specifically expressed genes drive the expression of the tetracycline-controlled transactivator (*tTA*). The expressed heterologous transactivator then activates the expression of the lethal effector gene hid^{Ala5} (Bergmann *et al*, 1998) and leads to complete embryonic lethality in *D.* melanogaster. To generate suppressible, dominant lethality in medfly and at the same time restrict the effects of lethality to embryos, a direct transfer of the sterility system from *D. melanogaster* (Horn & Wimmer, 2003) to medfly was previously tried. The genomic integration of the driver construct carrying the *sry* α P/E from *D. melanogaster* into medfly was successful, but none of the transgene insertions expressed the system activator *tTA* at a detectible level (Schetelig *et al*, 2008). We therefore concluded that the cellularization-specific P/E from *D. melanogaster* is not functional in medfly and that endogenous P/Es have to be used to generate such a system.

Here, we report the development of the first transgenic embryonic lethality system for medfly using an early embryonic lethal transgene combination. When transgenic males carrying this system are mated to WT females, all progeny die during embryogenesis without the need of radiation. Due to the complete lethality in embryonic stages no fruit damage would occur from progeny of WT females mated to transgenic males and no transgenes would ingress into the wild population. Moreover, males carrying this system are highly competitive in laboratory and field cage tests. After successful evaluation, a combination of this new embryonic lethal medfly system with a sexing system will become a powerful tool to improve SIT programs.

Results

Isolation of cellularization-specifically expressed genes and their P/Es from medfly

The isolation of the medfly homologs of the cellularization genes *sry α* and *nullo* by degenerated primer PCR using an embryonic cDNA pool was not successful (Schetelig *et al*, 2007). Thus, we carried out PCR-based cDNA subtractions of different embryonic stages and identified several cellularization-specific genes (Fig. 1). The genes *C.c.-slow as molasses* (*C.c.-slam;* Fig. 1A), *C.c.-sub2_99* (Fig. 1B), *C.c.-CG2186* (Fig. 1C), *C.c.-serendipity α* (*C.c.-sry α;* Fig. 1D), *C.c.-sub2_63* (Fig. 1E), and *C.c.-sub2_65* (Fig. 1F) are expressed specifically during medfly blastoderm cellularization (Fig. 1x2). None of the genes show maternal expression or expression at later stages, except *C.c.-sub2_63* which is additionally expressed during germ band elongation (Fig. 1E3).

Germline transformation with driver and effector constructs

By inverse PCR, we isolated the P/Es from *C.c.-slam, C.c.-sub2_99, C.c.-CG2186, C.c.-sry α*, and *C.c.-sub2_63* containing about 0.4 to 1.9 kb of the complete 5`UTR and upstream sequences. The isolated P/Es were fused to the tetracycline-controlled transactivator gene *tTA* and used to engineer different driver constructs (*sl1-tTA*, *sl2-tTA*, *99-tTA*, *CG2186-tTA*, *sryα2-tTA*, and *63-tTA*) embedded into *piggyBac* vectors carrying polyubiquitin (PUb) driven DsRed as a germline transformation marker (Handler & Harrell, 2001). Additionally, three effector constructs were generated (*TREp-hid^{Ala5}*, *TREhs43-hid^{Ala5}*, and *>TREp-hid^{Ala5}>*) carrying the lethal factor *hid^{Ala5}* under control of either *P* element (Rorth, 1998) or *hsp70* basal promoters (Thummel & Pirotta, 1992) from *D. melanogaster*. In the *>TREp-hid^{Ala5}>* construct the lethality inducing transgene is flanked by *gypsy* insulator elements (> = *gypsy* element in 5´-3´ orientation), which should reduce the variable expression strength due to position effects (Sarkar *et al*, 2006). Except for *sl1-tTA*, all constructs carry a minimal *attachment P* (*attP*) site (Groth *et al*, 2004), which will potentially enable site-specific phiC31-integrase-mediated integration to modify transgenes at successfully evaluated genomic positions (Wimmer, 2005b).

Five driver constructs (*sl1-tTA*, *sl2-tTA*, *99-tTA*, *CG2186-tTA*, and *sryα2-tTA*) and all three effector constructs were used for germline transformation of medfly. For each construct we obtained transgenes of which we further analyzed a maximum of three independent lines (Fig. 2 and Fig. 3).

Early expression of *tTA* activates *hid^{Ala5}*-mediated lethality

We crossed twelve independent homozygous driver lines to five independent homozygous effector lines to generate 60 different combinations. From each combination, we collected eggs to visualize the early expressed *tTA* and the proapoptotic gene *hid^{Ala5}* by *in-situ* hybridizations. The lethal activity of each combination was checked by a second egg collection, which was counted for eggs and progeny. To describe the dimension of lethality we henceforth use the term “complete lethality” for 100%

lethality in laboratory experiments. Combinations that showed detectibly lower or no progeny were inbred to generate homozygous (for both driver and effector construct) lethality lines (LLs).

All LLs expressed *tTA* specifically during cellularization. However, due to the different P/Es as well as integration sites, the *tTA* expression strength varied and resulted in different expression strengths of *hid*^{Ala5} (Fig. 2 and Fig. 3). This resulted in variable efficiencies of the lethality system. LLs deriving from the same driver line showed similar expression levels of *tTA*. The P/Es *sl1* (Fig. 2A1-3) and *99* (Fig. 2C1-3) mediated only very weak expression of *tTA*, which subsequently could not induce detectable levels of *hid*^{Ala5} expression. The longer P/E region of *sl2* (1.9 kb) was able to drive *tTA* and indirectly *hid*^{Ala5} (Fig. 2B1-6), but the level of *hid*^{Ala5} expression was not high enough to drive complete lethality (Fig. 2). With the P/E *CG2186* we obtained a strong level of *tTA* expression during cellularization (Fig. 2D2), which started the expression of *hid*^{Ala5} during the cellularization stage (Fig. 2D5) and led to complete pupal lethality of LL #68 (Fig. 2).

Besides the finding that different P/Es or P/E regions act differently on *tTA* and the dependent *hid*^{Ala5} expression, also the integration site of the driver construct could influence the *tTA* expression (Fig. 3). Three independent lines, carrying the driver construct with the *sry α* P/E at different integration sites, expressed the *tTA* specifically but with different strength during cellularization (Fig. 3A2-C2). In line #65, a weak expression of *tTA* led to a late expression of *hid*^{Ala5} during germ band retraction, which was not able to drive complete lethality (Fig. 3). In contrast, the lines #66 and #67 express *tTA* strongly during cellularization (Fig. 3B2, C2), which activates *hid*^{Ala5} expression at the cellularization stage (Fig. 3B5, C5) and lead to complete L1 larval lethality for line #66 and complete embryonic lethality for line #67 (Fig. 3). Thus, a strong *tTA* expression seems to be important to start the *hid*^{Ala5} expression early enough to cause complete embryonic lethality.

In addition, the effector constructs with different basal *D. melanogaster* promoters or different integrations of the same effector construct influence the levels of *hid*^{Ala5} expression and lethality. The effector constructs *TREp-hid*^{Ala5} and >*TREp-hid*^{Ala5}>, carrying the *p*-basal promoter, were able to express *hid*^{Ala5} in medfly after activation through the twelve independent driver lines, but did not cause complete lethality in 36 different LLs (data not shown). Interestingly, the effector construct *TREhs43-hid*^{Ala5}, which carries the basal promoter (43 bp) of *D. melanogaster hsp70*, showed differences in the expression strength of *hid*^{Ala5} depending on the integration site of the construct. In comparison to the larval or embryonic lethal lines #66 or #67, which are derived from the effector line *TREhs43-hid*^{Ala5}_F1m2, the *hid*^{Ala5} expression in #29 and #72 deriving from *TREhs43-hid*^{Ala5}_F1m1 started at later stages (Fig. 3D6, E6) and was not sufficient to drive complete lethality at the embryonic, larval, or pupal stage. Thus, we can show that the choice of E/Ps as well as the integration site of both the driver and the effector is crucial to set up a successful embryonic lethality system.

Molecular and cytogenetic characterization of transgenic lines

Transgenic driver and effector lines were preliminarily screened for homozygous condition by fluorescence patterns and intensity. Southern blots indicated single copy integrations of driver and

effector constructs of the lines *sryα2-tTA*_F4m1, *sryα2-tTA*_M2m1, *TREhs43-hid^{Ala5}*_F1m1, and *TREhs43-hid^{Ala5}*_F1m2 (Fig. 4). Moreover, the correct *piggyBac*-mediated integrations at canonical *TTAA* target sites were verified by isolation of 5´ and 3´ insertion site sequences by inverse PCR (see Supplementary Information). Therefore, we know that differences in expression strength and functionality of the lethality system in different LLs are not a result of multiple insertions of the driver or effector constructs, but must be due to position effects. Furthermore, the integration sites of the driver and effector construct for LL #66 and LL #67 were mapped by chromosome spreads. We found the driver and the effector of LL #66 or LL #67 located on chromosome 5 at the positions 74B and 70B or 63B and 70B, respectively (see Fig. S1 and S2).

Maternal suppressibility of lethality

Maternal suppressibility was tested by adding tetracycline (Tc) or doxycycline (Dox) to the adult and/or the larval food. To identify the minimal concentrations of Tc needed to rear the LLs #29, #72, #66, #67, and #68, flies were bred on larval and adult media containing different concentrations of Tc. We defined the optimal Tc concentration in adult and larval medium for rearing as the lowest possible amount of Tc combined with the highest possible number of descendants. The LLs #72, #66, or #67 could be reared efficiently on adult medium containing 10 µg/ml Tc and line #29 even on 1 µg/ml Tc. All LLs could be reared on larval medium containing 1 µg/ml Tc, except for #68 (10 µg/ml Tc). When reared on larval medium lacking Tc all lines showed a reduction of progeny. In addition all lines and WT showed delayed ovary development and a 5-7 days postponed egg laying when reared on larval medium containing 300 µg/ml Tc. This indicates the importance of reducing the Tc concentrations to a minimum for the efficient rearing of medfly lines.

Rearing LLs #66 and #67 on adult food containing 100 µg/ml Dox resulted in maternal suppression of the lethality, even without Dox in the larval food. Hatching and pupation rates were comparable to WT flies reared on adult and larval food without Dox. Thus, Dox is able to maternally suppress the embryonic lethality. However, eclosion rates were still reduced when Dox was used in adult food only (data not shown).

Efficiency, competition and reversibility tests

During medfly SIT programs, irradiation sterilized males are released into affected areas and mate to WT females, which leads to infertile matings. Ideally all progeny die as embryos to exclude damage to fruits from larval feeding. To show the efficacy and time point of lethality for the newly generated LLs, transgenic males (homozygous for driver and effector) from lines #29, #72, #66, #67, #68, or WT were crossed to WT females, respectively (Fig. 5A). For the LLs #29 and #72, about 20% of the eggs survived to become L1 larvae, whereas pupae and adult progeny were highly reduced. Crossings with males from #66, #67, and #68 showed complete pupal lethality but varying larval and embryonic lethality. Only 0,8% of the laid eggs from the #66-crossing hatched and all of those died during L1 larval stage. Line #67 showed the desired complete embryonic lethality.

An ideal line for releasing purposes should be embryonic lethal, but should also be competitive. We therefore did competition tests with lines #66 and #67 (Fig. 5B). WT females were crossed to WT males and transgenic males in different ratios (1:1:1, 1:1:3, 1:1:5, 1:1:9). The reduction of progeny compared to WT-only controls showed that both lines are highly competitive. Remarkable is the higher fertilization success of #67 males compared to WT males starting from ratio 1:1:5. For the ratio 1:1:9 an overall progeny rate of only 0,4% was measured. At the same time a WT control at ratio 1:10:0 gave only little reduction of overall progeny (Fig. 5B, ++). Thus, transgenic males from line #66 and #67 performed in laboratory competition tests comparable or even better than WT males. Progeny from all competition tests were identified as non-transgenic individuals by fluorescent microscopy, which additionally indicated the complete lethality of line #66 and #67. Interestingly, all lines deriving from the effector line *TREhs43-hid*^{Ala5}_F1m2 (#66, #67, and #68) partially lack anterior orbital bristles, which does obviously not interfere with the mating success of these transgenic males. In addition to laboratory tests, field cage tests with #67 males showed a comparable or even better competitiveness than WT (see Supplementary Information).

To test for reversible sterility of lines #66 and #67, adults were reared on Tc-containing medium (10 µg/ml) for two days (Fig. 5C). After transfer to Tc-free medium the rate of progeny decreased in five days to 0%. The sterility could be reversed by retransfer of the adults to Tc-containing medium. The reduced rate of progeny after this procedure could be due to a slight irreversible effect of the lethal system or to the advanced age of flies as shown in other studies (Scolari *et al*, 2008).

Discussion

In this study we describe the first transgenic embryonic lethality system for the insect pest *C. capitata* that causes complete reproductive sterility without the need of radiation. The use of a newly isolated early embryonic P/E of medfly makes a conditional embryonic lethality without larval hatching possible. The described embryonic lethality system has the advantage of eliminating the radiation process, a possible release of insects at any life-cycle stage and an expected fitness benefit of transgenic males over radiated males. Other transgenic lethality systems without the need of radiation could reduce the eclosion rate of flies, but as larvae are still produced, larval damage to targeted food would still be present (Gong *et al*, 2005).

In the presented embryonic lethality system, the tTA expression mediated by cellularization-specific P/Es not only depends on the P/E itself but also on the integration sites in the genome. These differences in expression led to a variety of lethality levels in the 60 tested transgene combinations. Interestingly, the basal promoters of the *P* element (*p*) and *hsp70* (*hs43*) from *D. melanogaster* were both able to drive the lethal factor *hid*^{Ala5} after *TRE*-mediated activation by tTA in *C. capitata*. However, effectors containing the *p* basal promoter could not promote complete lethality, whereas

systems with effectors containing *hs43*, which gave no functional transgenes in *D. melanogaster* (Horn & Wimmer, 2003), caused complete lethality in medfly at different stages of development. This demonstrates that even a molecularly well-constructed system is highly dependent on the specific P/Es used and on the integration sites of the transgenes.

The additional finding that both constructs of the lethal and competitive lines #66 and #67 are located on chromosome 5 has several advantages. First, this line can be combined with different well-established systems carried also on chromosome 5: e.g. a phenotypic marker system (Niyazi *et al*, 2005) or part of a genetic sexing system, as in genetic sexing strain (GSS) *Vienna-8* (Franz, 2005). The advantage of having different systems on chromosome 5 is a simplified quality control during rearing procedures. Second, the embryonic lethality line brings about two fluorescent markers (DsRed and EGFP), which are not only helpful during quality control but could also help for monitoring processes. Third, our constructs introduced *attP* sequences, which will allow to site-specifically modify this competitive embryonic LL by using the integrase system from phage *phiC31* (Groth *et al*, 2004). Possible applications will be the deletion of *piggyBac* ends to further increase the safety of transgenes or insertion of recently developed sperm markers for improved monitoring (Scolari *et al*, 2008).

To suppress lethality during mass rearing, Tc or Dox can be used as a supplement in the food. In laboratory assays, we were able to reduce the Tc concentrations in adult and larval media to 1 µg/ml at larval and to a minimum of 10 µg/ml at adult stages. Using these Tc concentrations for rearing, we could not detect any hid^{Ala5} expression during embryogenesis. In addition, we investigated a delayed ovary development and postponed egg laying when 300 µg/ml Tc was used in larval medium. Dox at 100 µg/ml in the adult food was sufficient to maternally suppress the embryonic lethality without the need for Dox in the larval food. Dox concentrations were not further optimized to a minimum. However, eclosion rates were reduced when Dox was used in the adult food only, which suggests the need for some Dox in the larval food. These parameters will be important for further evaluation and optimization of the embryonic lethality system in mass rearing.

Both lines #66 and #67 have shown high competitiveness to WT in laboratory and line #67 also in field cage tests. These transgenic LLs can now be used to evaluate the fitness costs of transgenic lethality compared to radiation-based sterility. The current 100-fold inundation of affected areas with sterilized males could be reduced with competitive LLs to lower amounts and at the same time embryonic lethality can be maintained. We could show that the embryonic LL #67 is a “100% sterility”-system. Such systems are required for preventional SIT programs in California or Florida (Dowell *et al*, 2000) and desired for every other pest management program. In addition, the use of such transgenic sterility systems can increase the safety of the mass-rearing process during operational SIT programs, since accidental releases could not lead to infestations of the environment (Wimmer, 2005a).

Conclusion

The first successful transfer of the *Drosophila* proof-of-principle embryonic lethality system to an agricultural pest, the medfly *C. capitata*, represents despite the hard work a straightforward approach that can be applied to further pest insects. We show that complete embryonic lethality in *C. capitata* is possible and that the responsible transgenes do not reduce competitiveness. The 100% embryonic lethal system is proposed as an alternative to reproductive sterility achieved by radiation for pest management programs based on the Sterile Insect Technique.

Methods

Details on medfly samples, isolation of cellularization-specifically expressed genes, inverse PCR, plasmid construction, germline transformation, Southern hybridization, *in-situ* hybridization, chromosome spreads, field cage tests, and Tc-optimization tests are described in the Supplementary Information. Primers are shown in Table S1. Complete cDNA sequences are deposited to GenBank (Accession numbers in preparation).

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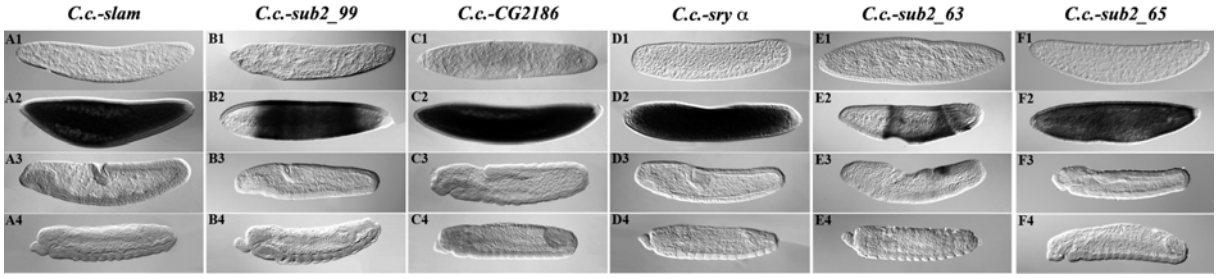


Figure 1. Medfly genes expressed specifically during cellularization. Gene expression is shown by WMISH with gene specific RNA probes for different stages during embryogenesis: early blastoderm (x1), cellularization (x2), germ band elongation (x3) and germ band retraction (x4). The genes *C.c.-slam* (Ay), *C.c.-sub2_99* (By), *C.c.-CG2186* (Cy), *C.c.-sry α* (Dy), *C.c.-sub2_63* (Ey), and *C.c.-sub2_65* (Fy) are strongly expressed during cellularization (x2). *C.c.-sub2_63* showed also expression during germ band elongation (E3). Gene names used in Schetelig et al. (2007) correspond as follows: *sub1_68 = sub1_24 = C.c.-slam; sub1_478 = C.c.-CG2186*.

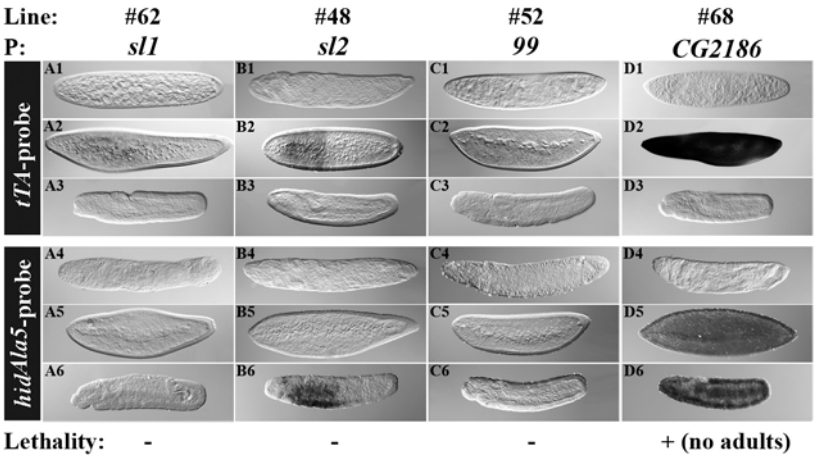


Figure 2. *tTA* and *hid*^{Ala5} expression depends on different P/Es. Expression of *tTA* and *hid*^{Ala5} is shown by WMISH performed on embryos from medfly lines carrying both driver and effector constructs in homozygous condition. Embryogenesis stages: blastoderm (x1 and x4), cellularization (x2 and x5), and germ band elongation/retraction (x3 and x6). The lines carry driver constructs with different P/E (P) driving the *tTA*. The depicted lines are representative for independent lines (three for *sl1*, two for *sl2*, three for *99*, and one for *CG2186*) carrying the respective driver construct. All presented lines derive from the effector line *TREhs43-hid*^{Ala5}_F1m2 and were reared on Tc-free adult food. 100% lethality in lab tests is indicated with + and the stage of complete lethality is indicated in brackets.

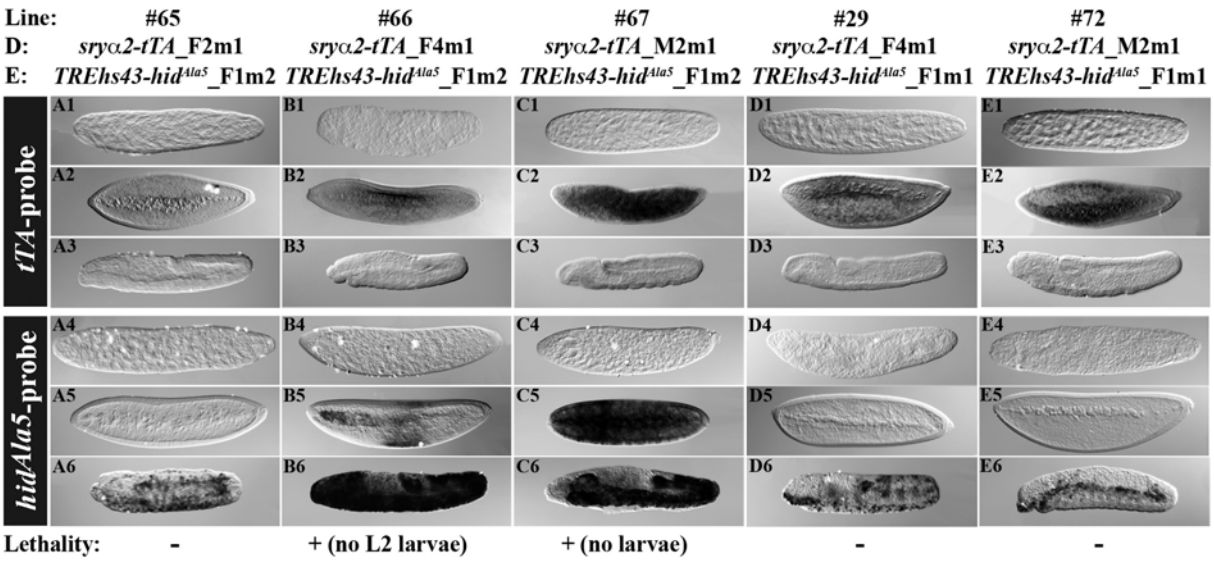


Figure 3. *tTA* and *hid*^{Ala5} expression depending on different integration sites. The expression of *tTA* and *hid*^{Ala5} is shown by WMISH performed on embryos from medfly combinations (comb.) carrying both driver (D) *sryα2-tTA* and effector (E) *TREhs43-hid*^{Ala5} in heterozygous conditions. Embryonic stages are as in Fig.2. The flies were reared on Tc-free adult food. 100% lethality in lab tests is indicated with + and the stage of complete lethality is indicated in brackets.

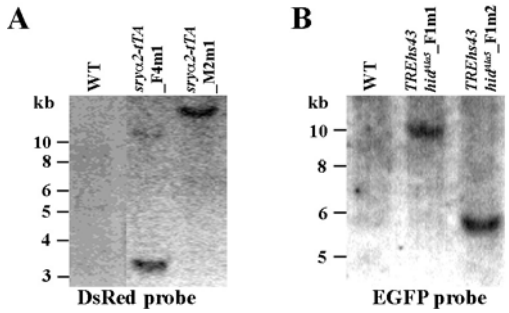


Figure 4. Southern hybridizations. (A and B) *Bam*HI-digested genomic DNAs isolated from indicated medfly lines were hybridized with DsRed (A) or EGFP (B) probes, respectively (see Supplementary Information). WT genomic DNA was used as a control for both. A single band in each lane indicates single integrations of the transgenes.

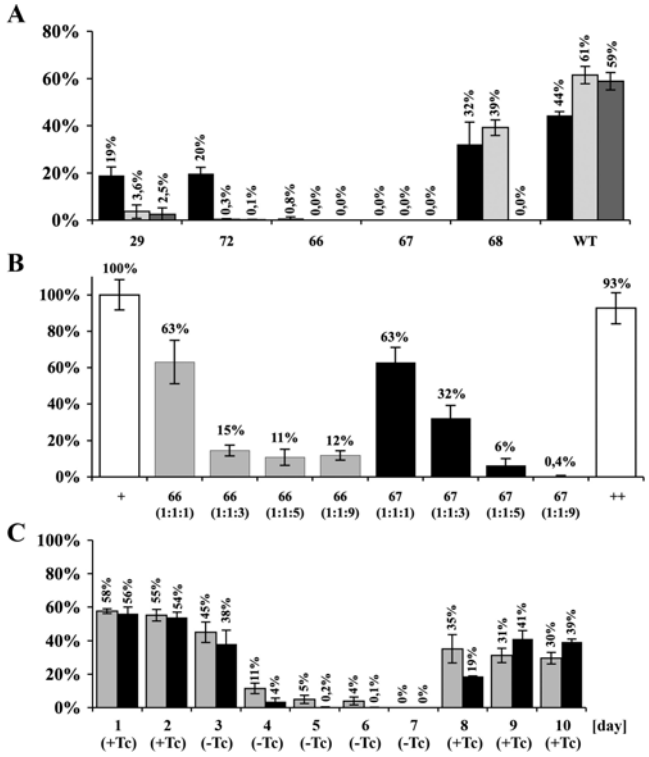


Figure 5. Efficiency, competition and reversibility tests with strains carrying the suppressible lethality system. (**A**) Efficiency test: Tc-free adult and larval food was used. In four independent repetitions, virgin WT females were crossed directly after eclosion to homozygous males from LLs #29, #72, #66, #67, #68 or WT, respectively. Four days later a 24 h egg collection was taken from each crossing. Shown are the combined data of the four repetitions. Total hatched L1 larvae 48h after egg collection (black bars), total pupae (light grey bars), and total adults (grey bars) were counted and are shown in relation to the total number of eggs (total egg number: n (#29) = 1481; n (#72) = 4330; n (#66) = 2278; n (#67) = 2058; n (#68) = 1914; n (WT) = 1712). Due to difficulties in the larval count, the number of surviving larvae might be an under-representation. The SD of four repetitions is indicated. Differences between repetitions are non-significant (ns), as shown by t-tests (Table S2). (**B**) Competition for virgin WT females: 15 WT females and 15 WT males were placed together with different numbers of LL #66 (grey bars) or LL #67 (black bars) males (15 (1:1:1) – 135 (1:1:9)). For control matings (white bars), 15 virgin WT females were crossed with either 15 WT males (+) or 150 WT males (++). Two independent crossings were performed for each ratio. Six 24 h egg collections were obtained from each crossing and the number of adult progeny was recorded. Numbers are normalized to the positive control (+). Adult progeny were verified by fluorescence light microscopy as WT or transgenic offspring. The SD of two repetitions is indicated. Differences between repetitions are ns, as shown by t-tests (Table S2). (**C**) Reversible lethality: three day old flies from LL #66 (grey bars) and LL #67 (black bars) were reared on Tc-containing food (+Tc; 10 µg/ml) for two days, transferred to Tc-free medium (-Tc) for five days and transferred back to Tc-containing food for three days. Progeny of 24 h egg lay intervals were monitored (embryos from Tc-containing or Tc-free adult medium were reared on 1 µg/ml Tc-containing or Tc-free larval food, respectively). The ratio of adults to laid eggs is shown. For comparison, the ratio of eclosed adults to laid eggs in WT was in a range of 54% - 74% under our rearing conditions (not shown). Two repetitions of the time series were performed for both transgenic lines. The SD of two repetitions is indicated. Differences between repetitions are ns, as shown by chi-test (Table S2).

Supplementary Information

Methods

Medfly samples

WT and transgenic medfly lines were maintained under standard rearing conditions (Saul, 1982). The WT strain *Egypt-II* (*EgII*) was obtained from the FAO/IAEA Agriculture and Biotechnology Laboratory (Entomology Unit, Seibersdorf, Austria). Laboratory tests for efficiency, competition and reversibility of the lethality system were performed in 15 x 15 x 20 cm acrylic cages. The Argentinean wild type strain (Arg; from Mendoza, Argentina) was reared for about 20 generations under relaxed artificial rearing conditions (Entomology Unit, Seibersdorf, Austria) and then used for the field cage tests.

Isolation of cellularization specific expressed genes

The Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, Heidelberg) was used to isolate fragments of the following genes expressed specifically during cellularization as described (Schetelig *et al*, 2007): *C.c.-slam*, *C.c.-sub2_99*, *C.c.-CG2186*, *C.c.-sub2_63*, and *C.c.-sub2_65*. An EST fragment of the medfly cellularization gene *serendipity* α (*C.c.-sry* α) was received from Dr. Ludvik Gomulski, Pavia. By RACE, 5´ and 3´ends of cellularization specific genes were isolated using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Heidelberg) and gene specific primers.

Inverse PCR

Inverse PCR was performed to get the 5´ regions of genes specifically expressed during cellularization: 1.5 µg of medfly WT genomic DNA was digested for 24 h; restriction fragments were precipitated and self-ligated in a volume of 500 µl at 16°C for 24 h; PCR was performed on circularized fragments by using primer sequences in opposite orientation within the 5´UTR or ORF of the genes. First, PCRs (1 min at 95°C; 6 cycles of 30 sec at 94°C, 45 sec at 66°C (-2°C each cycle), 6 min at 68°C; 25 cycles of 30 sec at 94°C, 45 sec at 54°C, 6 min at 68°C; and 6 min at 68°C) for *C.c.-slam, C.c.-sub2_99, C.c.-CG2186, C.c.-sry* α or *C.c.-sub2_63* were performed on *Fsp*BI, *Nde*I, *Cvi*AII, *Pvu*I *or Acl*I cut genomic DNA with the primer pairs mfs-77/-79, mfs-85/-108, mfs-170/-172, mfs-159/-161 or mfs-83/-104, respectively, using BD Advantage 2 PCR (BD Biosciences, Heidelberg). Second, the obtained PCR products were diluted 1:50 with ddH_20 and nested PCRs with primer pairs mfs-78/-80 (*C.c.-slam*), mfs-160/-162 (*C.c.-sry*α) or mfs-171/-173 (*C.c.-CG2186*) were performed (1 min at 95°C; 22 cycles of 30 sec at 94°C, 45 sec at 54°C, 6 min at 68°C; and 6 min at 68°C) using 5 µl of the dilution and the BD Advantage 2 PCR Kit (BD Biosciences, Heidelberg). PCR products from first (*C.c.-sub2_99* and *C.c.-sub2_63*) and nested PCRs (*C.c.-slam, C.c.-sry* α and *C.c.-CG2186*) were cloned into pCRII vectors (Invitrogen, Karlsruhe) and sequenced.

To localize the integration sites of *piggyBac* vectors, inverse PCR was performed with primers and protocols as described (Horn *et al*, 2003). Sequences flanking *piggyBac* insertions are shown in this Supplementary information.

Two-step cloning procedure

Generally we compose our constructs in the cloning shuttle vector pSLfa1180fa. From the shuttle vectors the constructs can be easily placed in transformation vectors which carry *Fse*I and *Asc*I sites (*fa*-sites (Horn & Wimmer, 2000)).

Shuttle vectors

The pSL*af_attP-sl2-tTA_af* (#1231), pSL*af_attP-63-tTA_af* (#1232), pSL*af_attP-99-tTA_af* (#1234), pSL*af_attP-sryα2-tTA_af* (#1236) and pSL*af_attP-CcCG2186-tTA_af* (#1237) carry a 52 bp *attP* site (Thorpe *et al*, 2000). #1231, #1232 or #1234 was created by ligating annealed *attP* primers (mfs-201/-202) in the *Eco*RI cut pSL*af_sl2-tTA_af* (#1210), pSL*af_63-tTA_af* (#1211) or pSL*af_99-tTA_af* (#1212), respectively. #1236 or #1237 was created by ligating annealed *attP* primers (mfs-203/-204) in the *Nco*I cut pSL*af_sryα2-tTA_af* (#1225) or pSL*af_CG2186-tTA_af* (#1226), respectively.
#1210, #1211 or #1212 was created by ligating the *Eco*RI-*Xba*I cut *sl2* fragment (a 1.9 kb 5´-region of the gene *C.c.-slam*), the *Eco*RI-*Eco*31I cut 63 fragment (a 1.2 kb 5´-region of the gene *C.c.-sub2_63*) or the *Eco*RI-*Xba*I cut *99* fragment (a 0.7 kb 5´-region of the gene *C.c.-sub2_99*), amplified by PCR on genomic DNA with primer pairs mfs-141/-113, mfs-142/-143 or mfs-131/-133, in the *Eco*RI-*Xba*I cut pSL*af_tTA_af* (#1215), respectively. #1225 or #1226 was created by cloning the *Nco*I-*Xba*I cut *sryα2* fragment (a 1.6 kb 5´-region of the gene *C.c.-sry α*) or the *Nco*I-*Eco*31I cut *CG2186* fragment (a 1.2 kb 5´-region of the gene *C.c.-CG2186*), amplified with primer pair mfs-189/-188 or mfs-190/-191, in the *Nco*I-*Xba*I cut #1215, respectively. #1215 was generated by cloning a 1.5 kb *Xba*I-*Hind*III cut *tTA-SV40* fragment from pTetOff (Clontech, CA) in the *Xba*I-*Hind*III cut pSLfa1180fa (Horn & Wimmer, 2000).

Transformation vectors

The driver construct p*Bac{sl1-tTA_PUb-DsRed}* (*sl1-tTA*) was generated by ligating the *Bgl*II/*Xba*I cut *sl1* (a 0.4 kb 5´-region of the gene *C.c.-slam* amplified with primer pair mfs-112/-113 from genomic DNA) and the *Xba*I/*Bgl*II cut *tTA-SV40* (a 1.5 kb region amplified with primer pair mfs-110/-111 from pTetOff) in the *Bgl*II site of pB[PUbDsRed1] (Handler & Harrell, 2001).
The driver constructs p*Bac{f_attP-sl2-tTA_a_PUb-DsRed}* (*sl2-tTA*), p*Bac{f_attP-63-tTA_a_PUb-DsRed}* (*63-tTA*), p*Bac{f_attP-99-tTA_a_PUb-DsRed}* (*99-tTA*), p*Bac{f_attP-sryα2-tTA_a_PUb-DsRed}* (*sryα2-tTA*) or p*Bac{f_attP-CG2186-tTA_a_PUb-DsRed}* (*CG2186-tTA*) were generated by ligating the *Fse*I-*Asc*I fragment *attP-sl2-tTA*, *attP-63-tTA*, *attP-99-tTA*, *attP-sryα2-tTA* or *attP-CG2186-tTA* from #1231, #1232, #1234, #1236 or #1237 in the *Fse*I-*Asc*I cut p*Bac{fa_PUb-DsRed}* (#1200 (Scolari *et al*, 2008)), respectively.

The effector constructs p*Bac{fa_attP_f_TREp-hid^{Ala5}_a_PUb-EGFP}* (*TREp-hid^{Ala5}*) or p*Bac{fa_attP_f_TREhs43-hid^{Ala5}_a_PUb-EGFP}* (*TREhs43-hid^{Ala5}*) were generated by cloning the hybridized primers mfs-211/-212 in the *Xma*JI site of p*Bac{faf_TREp-hid^{Ala5}_a_PUb-EGFP}* (#1207) or p*Bac{faf_TREhs43-hid^{Ala5}_a_PUb-EGFP}* (#1208), respectively. #1207 or #1208 were created by ligating the *Asc*I fragments *TREp-hid^{Ala5}* (5.0 kb) or *TREhs43-hid^{Ala5}* (4.9 kb) from pSLfa_*TREp-hid^{Ala5}*_fa or pSLfa_*TREhs43-hid^{Ala5}*_fa (Horn & Wimmer, 2003) in the *Asc*I site of p*Bac{fa_PUb-EGFP}* #1201 (Scolari *et al*, 2008), respectively. The effector construct p*Bac{>fa_attP_f_TREp-hid^{Ala5}_a>_PUb-EGFP}* (*>TREp-hid^{Ala5}>*) was generated by ligating the *Asc*I-fragment *attP_f_TREp-hid^{Ala5}* from *TREp-hid^{Ala5}* in the *Asc*I-site of p*Bac{>fa>_PUb-EGFP}* (Scolari *et al*, 2008).

Germline transformation

Germline transformation experiments were performed by microinjection of *piggyBac* constructs (500 ng/µl) together with the p*hspBac* transposase helper plasmid (200 ng/µl) (Handler & Harrell, 1999) into WT embryos as described by Handler and James (Handler & James, 2000) with the following exceptions: injected eggs were covered with Voltalef 10S oil (Lehmann & Voss, Hamburg, Germany), placed at 28°C in parafilm closed Petri dishes with watered Whatman paper in the lid; neither eggs, larvae or pupae were heat shocked.

The vectors *sl1-tTA*, *sl2-tTA*, *99-tTA*, *sryα2-tTA*, *CG2186-tTA*, *TREp-hid^{Ala5}*, *TREhs43-hid^{Ala5}*, or *>TREp-hid^{Ala5}>* were injected into 600 embryos of which 260, 140, 160, 54, 83, 28, 63, or 52 survived to adulthood, respectively. Four female crossings (two to 25 G_0 females crossed to 15 WT males; F1 – F4) and four male crossings (two to 25 G_0 males crossed to 15 WT females; M1 – M4) were set up for each construct. G1 progeny were screened by epifluorescence for the expression of the *PUb-DsRed* or *PUb-EGFP*. Fluorescent progeny with different red or green patterns were backcrossed twice to WT to recognize possible multi-insertions and brought to homozygous conditions by inbreeding and checking fluorescence intensity. For screening of flies we used the fluorescence stereomicroscope Leica MZ16 FA with the filters DsRedwide (Ext. 546/12; Emm. 605/75) and EYFP (Ext. 500/20; Emm. 535/30).

To generate lethality lines (LLs), we crossed twelve homozygous driver lines and five homozygous effector lines in all possible combinations. We inbred those heterozygous combinations, which produced detectibly lower or no progeny, screened the progeny by fluorescent intensity for homozygous individuals and subsequently inbred these homozygous individuals to generate LLs homozygous for driver and effector construct.

Southern hybridization

Genomic DNA (~3-10 µg) from adult flies of different transgenic lines and the WT strain were digested with *Bam*HI (Roche, Mannheim) and separated on 1% agarose gels. DNA was transferred to nylon membranes (Hybond-N^+; GE Healthcare/Amersham, Little Chalfont) and immobilized by UV irradiation. Probe labeling and membrane hybridizations were performed according to the AlkPhos Direct kit (GE Healthcare, Little Chalfont). Signal detection was performed using CDP-star (GE Healthcare, Little Chalfont) followed by exposure for approximately 30 min on Kodak Biomax ML film.

The two probes for detecting DsRed or EGFP were amplified by PCR (2 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 53°C, 1 min at 72°C; 5 min at 72°C) from the constructs #1200 or #1201 with the primers mfs-333 and mfs-334 or mfs-335 and mfs-336, respectively.

In-situ hybridization

WMISH with RNA probes to embryos were performed as described (Davis *et al*, 2001). RNA antisense probes were prepared by *in-vitro* transcription with the DIG-RNA-Labeling Kit (Roche, Mannheim) from pCRII vectors (Invitrogen, Karlsruhe) containing subtraction cDNA fragments (p_*slam*, p_*99*, p_*CG2186*, p_*63*, p_*65*), an EST fragment (p_*sryα*) and the plasmids pBSK-*hid*^{Ala5} or pBSK-*tTA* (Horn & Wimmer, 2003). By PCR using the primer pair mfs-41/-42, cDNA fragments were amplified and transcribed with Sp6 polymerase. The plasmids p*BSK-hid*^{Ala5} or p*BSK-tTA* were linearized with *Cla*I or *Eco*RI and transcribed with T3 or T7 RNA polymerase, respectively.

Chromosome spreads

Chromosome *in-situ* hybridizations were performed with slight modifications as described (Zacharopoulou *et al*, 1992). Instead of horseradish peroxidase, the Biotin/Avidin system VECTASTAIN Elite ABC was used (Vector laboratories, Peterborough). Hybridization sites were identified and photographed using 60x oil objectives (Olympus phase contrast microscope) with reference to medfly salivary gland chromosome maps (Zacharopoulou, 1990). Squash preparations of salivary gland polytene chromosomes were made as described (Zacharopoulou *et al*, 1992). A DNA-probe recognizing *piggyBac* insertions, was prepared by PCR on genomic DNA from flies carrying a DsRed-marked *piggyBac* insertion (Handler & Harrell, 2001) with the primers DsRed_F and DsRed_R (1813 bp) using the Biotin High-Prime kit (Roche Diagnostics, Mannheim).

Field cage tests for mating competitiveness

Males from line #67 (non-irradiated or irradiated with 120 Gy, 48 hours before adult emergence) were competed against non-irradiated wild type Argentinean males for mating with Argentinean wild-type females in a field cage (FAO/IAEA/USDA, 2003). Pupae from the different strains/treatment were placed in emergence cages, and every 24 h adults were removed, sorted by sex, and placed in cages

with adult food (3:1, sugar:hydrolyzed yeast) and water for 6 d. Two days before the tests, flies were marked with a dot of water-based paint on the thorax (DEKA®, Unterhaching, Germany). In each field cage, three potted *Citrus aurantius* trees, 1.6 m in height with 1.5 m diameter canopy, were used as a mating arena. To follow the quarantine protocol, tests were performed in a greenhouse with controlled temperature (24-26ºC) and humidity (60-80%). On the day of the test, 20 sexually mature non-irradiated Argentinean males, 20 non-irradiated and 20 irradiated males from line #67 were released into the cage around 08:30. Approximately 20 min later, 20 virgin and sexually mature Argentinean females were released in the cage. Tests lasted 3 hours. Mating pairs were collected as they formed by allowing the pair to walk into a small vial. The type of mating couple was recorded and the proportion of mating was calculated for each mating type (see Fig. S3). After the couples separated, the males and females were identified and the mated females were grouped together depending on the type of mated male and transferred to small egging cages. Eggs were collected for five consecutive days and transferred to small Petri dishes with moist black filter paper. After four days of incubation, hatched larvae and un-hatched eggs were counted to determine the egg hatch for each mating type. Twelve replications of this test were carried out.

Optimization of suppressor concentrations for rearing

Starting from larval and adult media Tc-concentrations of 100 µg/ml, the minimal Tc concentrations for the lines #29, #66, #72, #67, and #68 were tested with WT as a control. First, flies were reared on adult medium containing 100 µg/ml Tc and eggs were collected on larval medium containing 0, 1, 3, 10, 30, 100, or 300 µg/ml Tc. Hatching, pupation and eclosion rates were recorded. Second, the adult medium Tc concentrations (1, 3, 10, 30, 100, or 300 µg/ml) were tested over three generations by using the optimized larval media concentrations (1 µg/ml for #29, #66, #72, and #67; 10 µg/ml for #68) in between the adult stages.

Freshly eclosed flies from the lines #66 and #67 were placed on adult food containing 100 µg/ml Dox. Four and six days after eclosion an egg collection was taken and the eggs reared on Dox-free larval medium. Hatching, pupation and eclosion rates were recorded and compared with data from WT (*EgII*) flies reared on Dox-free adult and larval food.

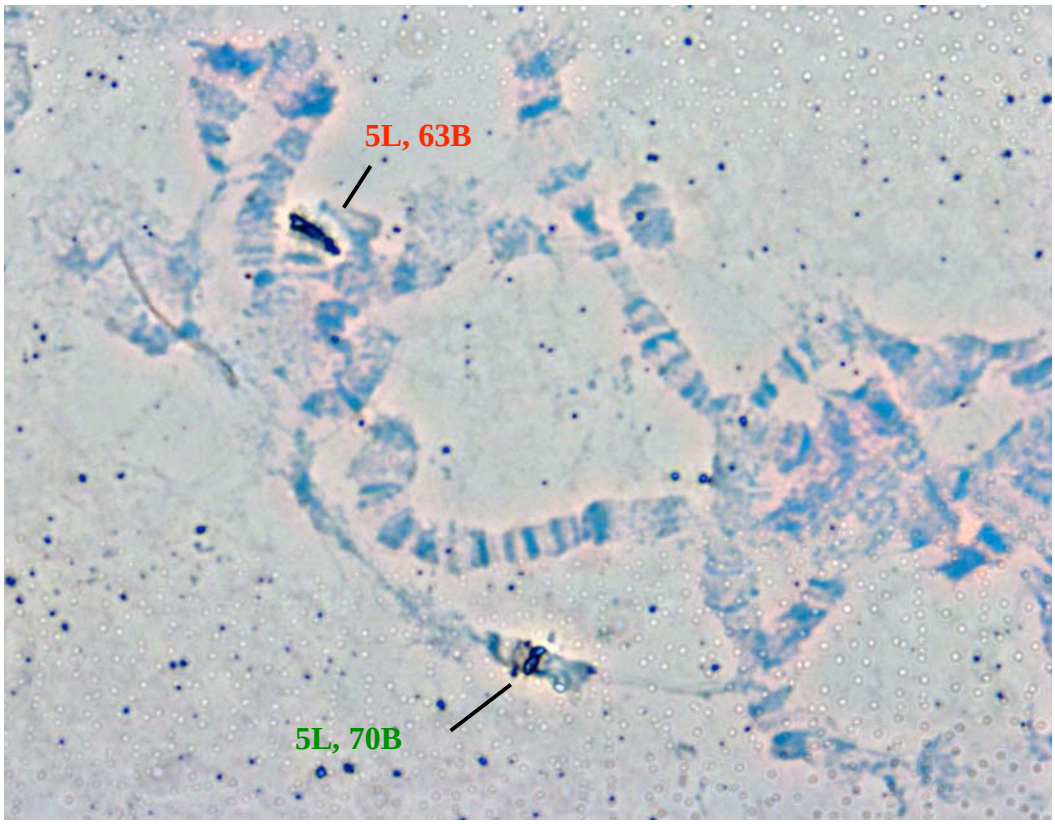


Figure S1. Chromosome *in-situ* hybridization on polytene chromosome spreads of embryonic LL #67. A double *in-situ* hybridization on spread chromosomes from LL #67 is shown. The integration sites of the driver construct *sryα2-tTA*_PUbDsRed was recognized at 5L_63B. The effector construct *TREhs43-hid*^{Ala5}_PUbEGFP was recognized at positions 5L_70B. The signals were assigned to the respective constructs by comparing the chromosomal locations with chromosomal *in-situ* hybridizations of LL #66, deriving from the same effector but different driver line.

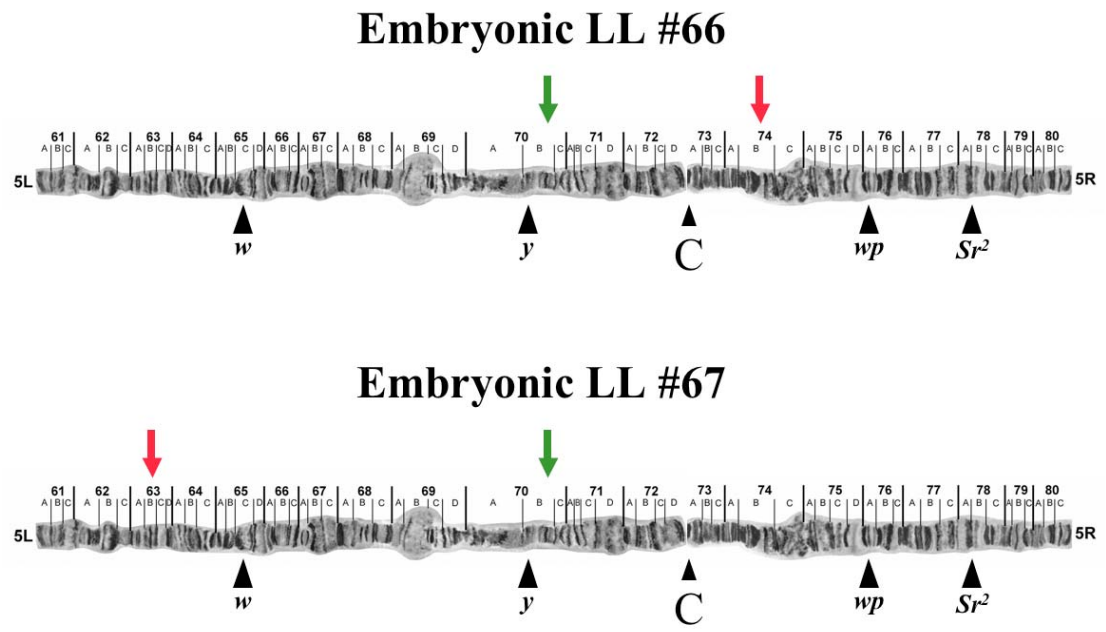
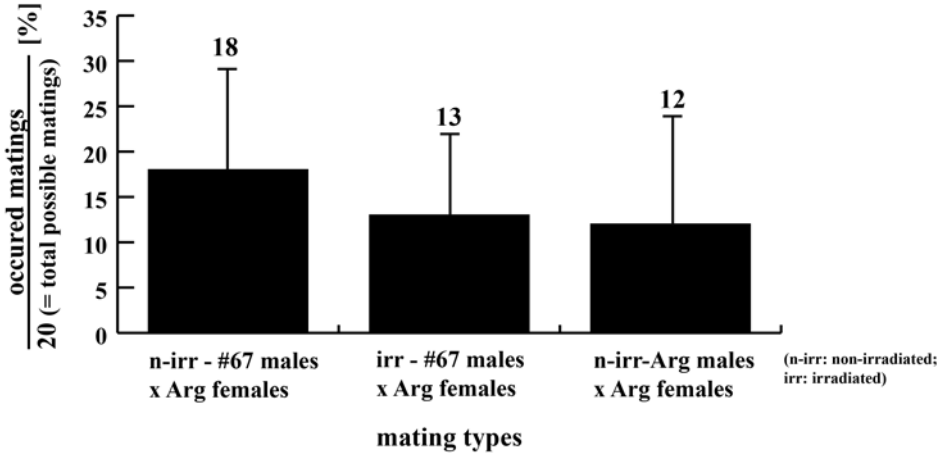


Figure S2. Schematic representation of chromosome 5 from the embryonic LLs #66 and #67. The two arrows show the integration sites of the effector construct *TREhs43-hidAla5*_PUbEGFP (green arrow) and the driver construct *sryα2-tTA*_PUbDsRed (red arrow) in respect to other genetic markers (arrowheads) on the fifth chromosome. The centromere is indicated as C.

A



B

Mating type	Number of eggs	Number of hatched larvae	Egg Hatch (%)
Line 67 (n-irr)♂ x Arg ♀	775	0	0
Line 67 (irr) ♂ x Arg ♀	887	0	0
Arg (n-irr) ♂ x Arg ♀	821	740	90.13

Figure S3. Mating competitiveness of line #67 in field cage tests. To test the competitiveness of the embryonic lethal line #67, 20 non-irradiated and 20 irradiated males from line #67 (120 Gy) competed with 20 non-irradiated wild type Argentinean (Arg) males for mating with 20 wild-type Argentinean females in a field cage. The males were marked with different colored water-based paints. Mating couples were taken out of the cage and the type of mating couple was recorded. Twelve replications were carried out. (**A**) The proportion of matings of each mating type was calculated by dividing the number of the occurred matings by the number of total possible matings (limited by the number of Argentinean females, n = 20). The proportion of matings was 18 ± 11 % for non-irradiated #67 males, 13 ± 9 % for irradiated #67 males and 12 ± 12 % for non-irradiated Argentinean males. The proportion of total matings over all twelve replications was 43 ± 5 % indicating an acceptable degree of sexual activity during the test period. The tests showed that non-irradiated and irradiated #67 males were at least as, if not more competitive than wild type non-irradiated Argentinean males. (**B**) Eggs and hatched larvae from each mating type were recorded and the egg hatch is shown. All matings of #67 males (regardless whether non-irradiated or irradiated) to wild type Argentinean females led to complete embryonic lethality.

In comparison to the complete lethality of strain #67 (descending from *EgII*) with or without irradiation, previous sterility tests with irradiated wild type *EgII* males (100 Gy) showed an egg hatch of 1.2 % (Franz, 2000). In addition, radiation induced sterility has been shown to be indirectly correlated to the competitiveness of the flies (Parker & Mehta, 2007).

Table S1. Primer sequences shown in 5´ to 3´ orientation.

mfs-41	GCGGATAACAATTTCACACAGGAAACAGCTATGAC
mfs-42	CCCAGTCACGACGTTGTAAAACGACGGCCAG
mfs-77	GGTTTTCGGATTCTTTGCAATTCACGATG
mfs-78	CGCTCTCGGAAAGATCAGTACG
mfs-79	CCACAGCCACCCGACGCCATTGG
mfs-80	CTTCGAGTGCTCCGTTGAAGATGATAGC
mfs-83	CGGAAGGCGCCAAATTGTCG
mfs-85	GCGAGAGGTTGTTGTATGTCCGGCAC
mfs-104	CATACGTTGAACAAGAGGCAGCCCGC
mfs-108	GTGTAATTGCTGGTCGGTCGACAC
mfs-110	CATAGAAGACACCGGGACCGATCCAG
mfs-111	GCATGCGGAGATCTAAGCTTGGTCGAG
mfs-112	GTCCGTAGAGATCTGCCGCAGCATTGTCC
mfs-113	AGCATTCTAGACATACTGGCCGGCGGAGC
mfs-117	GATCGGCCGGCCTTGGCGCGCCTA
mfs-118	GATCTAGGCGCGCCAAGGCCGGCC
mfs-131	GTCCGTGCGAATTCTGTATGCATAAGTCG
mfs-133	CGTAGGAGCTCTAGACATTTCTGTGATG
mfs-141	TCCTTATTGACCGTACGACCTTGTGGC
mfs-142	GTCCGTGCGAATTCTAAATTCTGAAAGCTATCTGG
mfs-143	TCAGGTCTCTCTAGACATTTTTTTTTTTAATTTTCACAATTCT
mfs-159	CACAGGAGCTGAAGTGCCAACTGATG
mfs-160	CTTCTGTGGTTACTTTTATGAGTTCGCCG
mfs-161	TCCGTCCGCCGTCATATTGG
mfs-162	TAGCCAGATTCCGTTTCACATTC
mfs-170	CTTCGAGTTCGATCACTGCACAATTC
mfs-171	AGTTTTCAGCCGCAGGCTTTCG
mfs-172	CGCATAAACTGGATGTGGCACGCC
mfs-173	ACATTGAAATGTAGTGAAATGGTGGCG
mfs-188	AGCATTCTAGACATATTGGATTTTCAATAAACAAGTATTTC
mfs-189	ACAGTCCATGGCTATCCGTGCTTTCGCTACATTTATC
mfs-190	ACAGTCCATGGCAGTGGTCACTCATCATCCTTCAAAATG
mfs-191	TCAGGTCTCTCTAGACATTTTGATAATTGAACACTTTACCACGCTC
mfs-201	AATTCGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGTC
mfs-202	AATTGACCCTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACG
mfs-203	CATGGGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGTCG
mfs-204	CATGCGACCCTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACC
mfs-211	CTAGGGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGTCG
mfs-212	CTAGCGACCCTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACC
mfs-333	GCTCCTCCAAGAACGTCATC
mfs-334	TGGTGTAGTCCTCGTTGTGG
mfs-335	GTAATACGACTCACTATAGGGCGGCGGTCACGAACTCCAG
mfs-336	GTGAGCAAGGGCGAGGAG
DsRed_F	CCACCACCTGTTCCTGTAGC
DsRed_R	TTGCCTTTCGCCTTATTTTAG

Table S2. Statistical analysis. ns = non-significant; - = the original data was 0 for all repetitions, statistics are therefore not possible. T-test and chi-test were performed as described in Sokal and Rohlf (Sokal & Rohlf, 1995).

(A) T-test for the efficiency tests

		stat	df	probability	significance
LL #29	L1 larvae	4,9448	2	0,1270	ns
	pupae	0,1624	2	0,8975	ns
	adults	0,1177	2	0,9254	ns
LL #72	L1 larvae	2,2391	2	0,2673	ns
	pupae	0,8260	2	0,5604	ns
	adults	1,4728	2	0,3797	ns
LL #66	L1 larvae	1,6379	2	0,3489	ns
	pupae	-	-	-	-
	adults	-	-	-	-
LL #67	L1 larvae	-	-	-	-
	pupae	-	-	-	-
	adults	-	-	-	-
LL #68	L1 larvae	0,6821	2	0,6188	ns
	pupae	0,2245	2	0,8593	ns
	adults	-	-	-	-
WT	L1 larvae	1,6911	2	0,3399	ns
	pupae	1,3158	2	0,4137	ns
	adults	1,9443	2	0,3024	ns

(B) T-test for the competition tests

	stat	df	probability	significance
+	0,9017	10	0,4182	ns
66 (1:1:1)	0,7000	10	0,5151	ns
66 (1:1:3)	1,0101	10	0,3588	ns
66 (1:1:5)	1,4861	10	0,1974	ns
66 (1:1:9)	1,0613	10	0,3371	ns
67 (1:1:1)	0,1265	10	0,9043	ns
67 (1:1:3)	0,0690	10	0,9477	ns
67 (1:1:5)	1,7320	10	0,1438	ns
67 (1:1:9)	1,4029	10	0,2196	ns
++	0,0987	10	0,9261	ns

(C) Chi-test for the reversibility tests

	stat	df	probability	significance
Day 1 (+Tc)	0,0008	1	0,9768	ns
Day 2 (+Tc)	0,0067	1	0,9348	ns
Day 3 (-Tc)	0,0152	1	0,9020	ns
Day 4 (-Tc)	0,0098	1	0,9213	ns
Day 5 (-Tc)	0,0120	1	0,9127	ns
Day 6 (-Tc)	0,0139	1	0,9060	ns
Day 7 (-Tc)	-	-	-	ns
Day 8 (+Tc)	0,0314	1	0,8593	ns
Day 9 (+Tc)	0,0088	1	0,9251	ns
Day 10 (+Tc)	0,0059	1	0,9389	ns

Sequences

Sequences flanking *piggyBac* insertions. Sequences are shown in 5´ to 3´ orientation in respect to the *piggyBac* insertion. Duplicated integration sites (**ttaa**) indicated in bold, enzyme restriction sites underlined (ggcc = *Hae*III; ccgg = *Msp*I).

sryα2-tTA_F4m1

ccggaaacacaaattaaaacacttcccgatgcataaaacataatgtcaaaagcaaaatttccgctgctttatccg
acaaaatcaaataaattatcaacaatgtttatttacccaaagcacattatttccacaagcctttcagtgcggctt
ctaattttactttctagatattttcacaaaatatattttgcactaattttggaaagacgaaatctacgaataaa
aacttcttcatcatatttctgaaagaaaatatttgtatacggcacgatctttccctataaatgcgcctctgaatc
aacgcagcgagtgcagtgcgttccatattttccataatgaaatttgtt**ttaa**…***piggyBac***…**ttaa**agccaacat
ccgg

sryα2-tTA_M2m1

ccggatatttaagtgataatcttttaaaccgtatctaaatacatattgtataaatataaataaattttgttacta
atttattta**ttaa**…***piggyBac***…**ttaa**tgttcattttctccgttaaaaaatctctaattacagttcttgcacgcc
tacgtttgatacttcctttgtaaacgtgtgactaataaaattcgccatcacgacgcagtacgcacttttaaccgt
taaacacattatttgtgtctacaaggtgaagaaaaaattcatcgaatcagttaataatacataaaatactcgcta
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tacgccgtgtagtaacgtccattctatcaaaccaacgtcgctttagccaaaccaacacccacccacacacacaca
ctcacaatgcccgttcaatctcccatcgcgattagcaatcgtgccattgaacatatcgatgatgtcgatcccttg
aaatatcatggccattgggaaccagttggtgttgcacgcgtacaaaatatccgg

TREhs43-hid^{Ala5}_F1m1

ggcctcctcaacagcttttctgtttgatacaacaaatctgtagagtcaaccttgagtaggggctgcagtagccaa
gaagttagcagtactattatacagaggggttgactggaccaagatctgttcggcactcggcagagaatgtctaag
aaatcattgtgtaataattgggatatatttttatcccagaattccgtattccatttgttagatttcctcacgttt
aaaaagctcataaagctcataacttcaactgtctcagcaacaaggcttcagaactaaggtatttgttagtttcaa
ctttacgccgcacatcgatataattaatcgtgcaaattgcgttattgatttgttgtcctcaattcttatgctccg
aacaaagtaaattctaaacaaacccaacttcagttaaacattcaacaaataaaagttacgttgggggaaatttat
ttatatatacgatattggcatatttaaagagggaccgttggaagagtaaaagcgtccctcattttacaatt**ttaa**
…*piggyBac*…ttaagagaggatatagaacatataaatccgagcgtatttccaacataattgtctcaaaagtttgg
atgtcttgaaactgagttcagaagagtgtcgtcagaaagggcttaacagtagatcggactcgtagctttcagtaa
ccttgcttaagattagtcatggagattaggaggtccgggctactagcagtttcaatgaatttcggttttagtaat
ggttgttgtcttgtggaactcactgaaggctgtttataacttacattggcc

TREhs43-hid^{Ala5}_F1m2

ggccaagtctggactgtaaggttggtgattaaaaatcgctcttatattgtcaacttatagaaacatatttttctt
tatgatcaatcaataatattctttccgagttccaaacgaccgtttgccatgagtttaccagctgactgtgaaatc
ttaaaccgaaaattgcactcatagact**ttaa**…***piggyBac***…**ttaa**aaatgtttgtatcggatacaccaagcgctt
ctgtaagttgtctcttctttggtggacgtcgttcggggacaatttttttctacctttttggacaaattctcgcgtg
atcaaaataaagagcaataaaaaactctctgctctactcacgttcgattagtgtttcaagtctttggagaagagt
gatggcagaaaggtacttgtcgagctggagagtatagttttgtcatctacaacggcatccaggtgttgatagagt
gccttttttccattccaaacgttctggtatgcattcctgtatgaagatggcacctttcaccaactcattaatatca
gttgtgcccgaaatgagcgcgaattgttggttagcactgagtttgccacgcagtaagcccttcgattccaagctt
ttcagctccttttctgtctcacgtaaggcattttcgatttgtgttggtacactgtcgtagagcactacttgatag
cccaccgaggcgaagagcatggaccatgaacgtccgataagaccgctaaatgttaaatattagtataaatttttgt
atttttttattgacgtttcacttgatattttcgttttctttgaaagtcacctgcccacaataccaactttgcat
tgactggcc

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3.5 Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae)

To further improve the SIT, the developed embryonic lethality system (3.4) might be modified to generate a transgenic sexing system for *C. capitata*. This should be achieved by introducing a female-specific spliced intron into the driver construct of the embryonic lethality system (3.4). Then the embryonic lethality should be activated in females only, because of the female-specific spliced intron. Only females would then be killed in conditions without the "system-suppressor" tetracycline in the food. Such a transgenic sexing systems would improve the following aspects of the SIT:

- Embryonic lethality of females to reduce the costs of mass-rearing
- In comparison to commonly used genetic sexing strains (GSSs) the possible amount of mass-reared males might be increased

Marc F. Schetelig and Ernst A. Wimmer.

Author contributions to the practical work:

Marc F. Schetelig: All experiments

STATUS: WORK IN PROGRESS.

Transgenic sexing system in *Ceratitis capitata* (Diptera: Tephritidae)

Marc F. Schetelig and Ernst A. Wimmer[§]

Department of Developmental Biology, Göttingen Center for Molecular Biosciences, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, Georg-August-University Göttingen, GZMB, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany. § To whom correspondence should be addressed. E-mail: ewimmer@gwdg.de; Tel.: ++49-551-392889; Fax: ++49-551-395416

Keywords: alternative splicing, female-specific spliced *transformer* intron, pest management, SIT, transgenesis

Introduction

The sterile insect technique (SIT) is an important component of area-wide pest management programs. This technology using radiation to sterilize mass-reared insects for release was extensively developed in the 1950s and is currently applied on six continents. *Ceratitis capitata* is the most important pest species fought by SIT programs. For *Ceratitis* SIT programs about 3.5 billion sterile male medflies are produced and released worldwide every week (Franz, 2005). Male-only mass-releases have been shown to be more effective in large-scale field trials by a factor of three- to five-fold compared to co-releasing sterile males and females (Rendon et al., 2004). This might be due to mating of sterile males to co-released sterile females rather than seeking out wild females. Moreover, co-releasing of medfly males and females increases the damage of fruits by additional oviposition and the costs during mass-rearing.

To generate medfly strains, which could be easily sex-separated even in mass scales, different genetic strategies have been followed. Sexing systems using classical genetics have been developed to kill medfly females during the rearing process and such strains are used routinely in medfly SIT programs. These medfly genetic sexing strains (GSSs) have a recessive, temperature sensitive lethal gene on an autosome and a complementing translocation of the wildtype gene locus to the Y chromosome (Franz, 2005). To kill the females of such GSSs, eggs are heatshocked during the rearing process, which induces lethality in the females in an early phase of embryogenesis. This early embryonic lethality is important to keep the cost of larval production during mass rearing low. However, disadvantages of the GSSs are fitness penalties due to the mutations and chromosome rearrangements required to construct the female-specific lethality. To possibly overcome these fitness problems, a transgenic female-specific lethality system for medfly was engineered using alternative splicing (Fu et al., 2007). The system uses a female-specifically spliced intron from the medfly *transformer* gene (*CctraI*), which is cloned into a dominant lethal genetic system (Gong et al., 2005). Thus, functionally lethal transcripts are only produced in females and it comes to the female-specific lethality. In laboratory assays, this combination had the advantage of killing all females and at the same time maintaining viable males. Such a system theoretically maintains about 30% more males than the existing GSSs (Gerald Franz, personal communication), even though behavior and fitness of these transgenic lines in field experiments and mass rearing are unknown so far. However, the published female-specific splicing system (Fu et al., 2007) is not able to generate embryonic lethality. Thus, female larvae and pupae develop and decrease the efficiency of the mass-rearing process.

In order to generate female-specific embryonic lethality, we try to combine a conditional embryonic lethality system from medfly (Schetelig et al., in preparation) with female-specific splicing by using the above described female-specifically spliced intron from the medfly *transformer* gene (Fu et al., 2007). The embryonic lethality system is based on the transmission of a transgene combination of driver and effector constructs that causes conditional embryo-specific lethality in the progeny. To limit

the effect of the transgenes to the embryonic stage, promoter/enhancers from cellularization-specifically expressed *C. capitata* genes drive the expression of the tetracycline-controlled transactivator (*tTA*), which then activates the expression of the lethal effector gene *hid*^{Ala5} (Bergmann et al., 1998) and leads to complete embryonic lethality in *C. capitata* (Schetelig et al., in preparation). Female-specific embryonic lethality should be generated by introducing the female-specifically spliced *transformer* intron into one of the constructs of the lethal transgene combination. In such transgenic sexing strains (TSSs), females should be killed during embryogenesis and males should survive and maintain their fitness to a higher degree than in GSSs or the previously described transgenic sexing systems.

Results

Generation of a female-specifically spliced driver construct used to generate transgenic lines

It had been shown that the position of the *CctraI* is crucial for a female-specific splicing system (Fu et al., 2007). To generate a situation similar to the functional one of Fu et al. (2007) the *CctraI* was inserted directly after the translational start site of the tetracycline-controlled transactivator (*tTA*) within the driver construct of the embryonic lethality system (Schetelig et al., in preparation) to generate *sryα2-CctraI-tTA* (Fig. 1). The driver construct *sryα2-CctraI-tTA* was injected into 500 embryos of which 231 larvae hatched and 108 survived to adulthood (54 G_0 females and 54 G_0 males). From 20 outcrossings to WT, three independent driver lines *sryα2-CctraI-tTA*_F1m1, *sryα2-CctraI-tTA*_M3m2, and *sryα2-CctraI-tTA*_MM1f1 were isolated.

Test for female-specific splicing

To check for the female-specific splicing of *CctraI* in the driver lines, heterozygous driver lines were mated to the homozygous effector line *TREhs43-hid*^{Ala5}_F1m2 (Schetelig et al., in preparation). If *CctraI* is female-specifically spliced, tTA is functionally produced exclusively in females, which would lead to the expression of the proapoptotic factor *hid*^{Ala5}. Thus, all females carrying the driver and the effector construct in heterozygous conditions should die. The heterozygous driver lines *sryα2-CctraI-tTA*_F1m1, *sryα2-CctraI-tTA*_M3m2, or *sryα2-CctraI-tTA*_MM1f1 were mated to the homozygous effector line *TREhs43-hid*^{Ala5}_F1m2, respectively (Table 1). A 20% decrease of females, heterozygous for driver and effector construct was observed for mating *sryα2-CctraI-tTA*_F1m1 to *TREhs43-hid*^{Ala5}_F1m2 in comparison to males heterozygous for driver and effector. This indicates a partial functionality of the female-specific killing system. For the other two crossings no differences in the ratio of males to females could be observed.

Discussion

To generate conditional female specific lethality for SIT programs, two components are needed: i) conditional embryonic lethality to kill individuals at the earliest stage possible, combined with ii) the ability to render the lethality female-specific. Therefore an embryonic lethal system (Schetelig et al., in preparation) was combined with the female-specifically spliced *CctraI* from medfly. Transgenes carrying the driver construct *srya2-CctraI-tTA* were established. Crossing these lines to an effector line did not lead to a complete lethality of females, which had been expected for all females heterozygous for the driver and effector construct. Instead only a 20% reduction of females heterozygous for driver and effector was observed for one transgene combination, whereas other combinations showed no effect. This could be due to position effects of driver or effector construct in the genome as previously shown for other systems (Schetelig et al., in preparation) and might be solved by analyzing other integration sites of drivers and/or effectors or different combinations thereof. Furthermore, the exact position of the intron within the transcript was shown to be crucial to obtain female-specific splicing in every female (Fu et al., 2007). Therefore, it might be worth to check *CctraI* at different positions within the driver or effector constructs.

Materials and Methods

Medfly samples

WT and transgenic medfly lines were maintained under standard rearing conditions (Saul, 1982). The WT line *Egypt-II* was obtained from the FAO/IAEA Agriculture and Biotechnology Laboratory (Seibersdorf, Vienna, Austria). The effector line *TREhs43-hid*^{Ala5}_F1m2 was previously described (Schetelig et al., in preparation).

Isolation of the female-specific spliced *transformer*-intron from *Ceratitis capitata*

The medfly *transformer* intron (*CctraI*) was isolated by PCR (2 min at 94 °C; 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 2 min at 72 °C; and 5 min at 72°C) on WT genomic DNA with the primer pair mfs-300 (GTAGGTCTCATGGTAATTTTAAAAGCATATTTTTTTCTTTGAAATTC)/ mfs-301 (AGTTCTAGAGACCTATAGATACCATAGATGTATGGATTAG) using the BD Advantage 2 PCR Kit (BD Biosciences, Heidelberg). The amplified 1.2 kb fragment was cloned into the pCRII vector (Invitrogen, Karlsruhe) to generate the construct pCRII-*CctraI_5* (#1284).

Two-step cloning procedure

Generally we compose our constructs in the cloning shuttle vector pSLfa1180fa (Horn and Wimmer, 2000). From the shuttle vectors the constructs can be easily placed in transformation vectors which carry *Fse*I and *Asc*I sites (*fa*-sites; (Horn and Wimmer, 2000)).

Shuttle vectors

The construct pSL*af_attP-sryα2-CctraI-tTA_af* (#1300) was created by ligating a 0.8 kb partial *CctraI*-fragment cut with *Xba*I from #1284 into the *Xba*I cut pSL*af_attP-sryα2-partialCctraI-tTA_af* (#1288). #1288 was generated by ligating two fragments into the *Nco*I/*Xba*I-cut vector backbone of pSL*af_attP-sryα2-tTA_af* (#1236; (Schetelig et al., in preparation)): i) a 0.5 kb partial *CctraI* fragment cut with *Eco*31I/*Xba*I from #1284; ii) the 1.6 kb *attP-srya2* fragment, isolated by PCR (2 min at 95 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 54 °C, 5 min at 72 °C; and 10 min at 72°C) on pSL*af_attP-sryα2-tTA_af* with the primer pair mfs-298 (TCGAATGGCCATGGGTGCCCCAACTGG)/ mfs-299 (GTAGGTCTCT*ACCA*TATTGGATTTTCAATAAACAAG) using *Pfu*-Polymerase (Fermentas, St. Leon-Rot) and finally cut with *Nco*I/*Eco*31I.

Transformation vectors

The driver construct p*Bac{f_attP-sryα2-CctraI-tTA_a_PUb-DsRed}* (#1301) was generated by ligating the *Fse*I-*Asc*I fragment *attP-sryα2-CctraI-tTA* from #1300 in the *Fse*I-*Asc*I cut p*Bac{fa_PUb-DsRed}* (#1200; (Scolari et al., 2008)).

Germline transformation

Germline transformation experiments were performed by microinjection of *piggyBac* constructs (500 ng/µl) together with the p*hspBa*c transposase helper plasmid (Handler and Harrell, 1999) (200 ng/µl) into WT embryos as described by Handler (Handler and James, 2000) with the following exceptions: injected eggs were covered with Voltalef 10S oil (Lehmann & Voss, Hamburg), placed at 28°C in parafilm closed Petri dishes with watered Whatman paper in the lid; neither eggs, larvae or pupae were heatshocked. Eclosed G_0 adults were screened for red fluorescence and 65 adults showed already red fluorescent dots in various tissues of the flies. Red fluorescent G_0 males and virgin females were backcrossed in eight groups of one individual to five virgin WT females (M1-M8) or five WT males (F1-F8), respectively. In addition, two matings of 23 G_0 males to 25 virgin WT females (MM1 and MM2) and two matings of 23 virgin females to 25 WT males (FM1 and FM2) were performed. G1 progeny were screened by epifluorescence for the expression of the *PUb-DsRed*, using the fluorescence stereomicroscope Leica MZ16 FA with the filter DsRedwide (Ext. 546/12; Emm. 605/75). One of the female crossings (F-1) and two of the male crossings (M-3 and MM1) had fluorescent progeny with different patterns. To recognize possible multi-insertions, one individual of each crossing was backcrossed to WT for two generations. No differences in the red fluorescent

pattern of these heterozygous progeny could be identified. Therefore, each line was inbred and the progeny screened for homozygous condition by fluorescence intensity. Subsequently supposingly homozygous flies of each line were crossed together to generate the lines *sryα2-CctraI-tTA*_F1m1, *sryα2-CctraI-tTA*_M3m2, and *sryα2-CctraI-tTA*_MM1f1.

Female-specific lethality test

To test for female-specific lethality, females from the heterozygous driver lines *sryα2-CctraI-tTA*_F1m1, *sryα2-CctraI-tTA*_M3m2, or *sryα2-CctraI-tTA*_MM1f1 were crossed to males from the homozygous effector line *TREhs43-hid*^{Ala5}_F1m2, respectively. About 400-800 progeny were counted for each crossing and checked for red and green fluorescence using the DsRedwide (Ext. 546/12; Emm. 605/75) and the EYFP filter (Ext. 500/20; Emm. 535/30).

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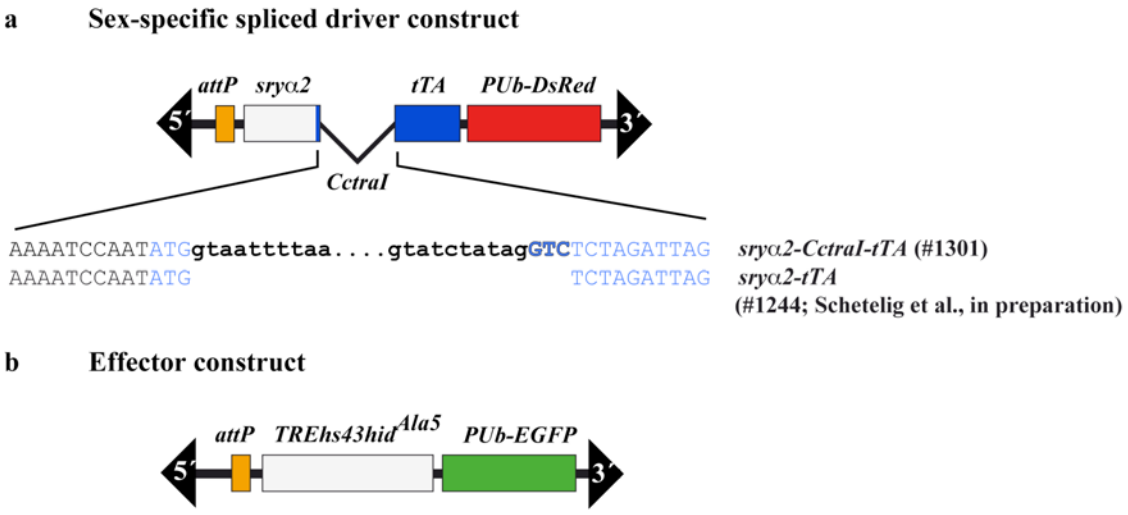

Figure 1. Binary expression system for embryonic female-specific lethality

Tetracycline-repressible binary expression system consisting of a female-specifically spliced driver construct and a lethality-inducing effector construct both integrated by *piggyBac* transposition into the genome of medfly. (**a**) The driver construct carries a cellularization-specific promoter (*sryα2*) driving tTA expression. The female-specifically spliced medfly *transformer* intron (*CctraI*) was inserted after the translational start site of *tTA* and the splice site was optimized according to functional female-specific spliced constructs (additional GTC shown in bold blue letters; (Fu et al., 2007)). Thus, functional tTA protein should be produced exclusively in females. (**b**) The effector construct carrying the *D. melanogaster* basal promoter (43 bp) of the *hsp70* and the phosphoacceptor-site mutant allele of the proapoptotic *D. melanogaster* gene *head involution defective* (*hid*^{Ala5}) can cause lethality in medfly when tTA binds to the *tTA responsive element* (TRE) (Schetelig et al., in preparation). Both driver and effector construct carry an *attachment P* (*attP*) site for potential future modifications.

Table 1. Test for female-specific lethality

Mating of			n (%)			
Heterozygous driver line		**Homozygous effector line**	**females [D, E]**	**males [D, E]**	**females [E]**	**males [E]**
sryα2-CctraI-tTA **_F1m1**	to	*TREhs43-hid^{Ala5}*_F1m2	80 (21,4%)	101 (27,0%)	96 (25,7%)	97 (25,9%)
sryα2-CctraI-tTA **_M3m2**	to	*TREhs43-hid^{Ala5}*_F1m2	192 (24,6%)	190 (24,3%)	201 (25,7%)	198 (25,4%)
sryα2-CctraI-tTA **_MM1f1**	to	*TREhs43-hid^{Ala5}*_F1m2	204 (25,4%)	199 (24,8%)	197 (24,5%)	203 (25,3%)

Different heterozygous female-specifically spliced driver lines (*sryα2-CctraI-tTA*_F1m1, *sryα2-CctraI-tTA*_M3m2, and *sryα2-CctraI-tTA*_MM1f1) were crossed to a homozygous effector line (*TREhs43-hid^{Ala5}*_F1m2). Progeny were sorted by fluorescent colors indicating the presence or absence of driver [D] and effector [E] constructs. The number of counted adult flies of each individual transgene combination (males [D,E], males [E], females [D,E] females [E]) and the percentage relative to the total number of flies is shown.

In general, progeny from medfly show a typical 50:50-ratio of males to females (data not shown). The reduction of females heterozygous for driver and effector constructs of the mating *sryα2-CctraI-tTA* _F1m1 to *TREhs43-hid^{Ala5}*_F1m2 was about 20% compared to males double heterozygous. For the other two combinations no detectable reduction was observed.

3.6 Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae)

In this part, a sperm marking systems for the medfly was established. Several lines with green or red fluorescent sperms were developed and successfully tested for their overall fitness. The system will help to improve the monitoring during medfly SIT programs and to foster the knowledge about reproductive biology in medfly.

Francesca Scolari◐, **Marc F. Schetelig**◐, Sabrina Bertin, Anna R. Malacrida, Giuliano Gasperi, and Ernst A. Wimmer (2008).
◐ = co-first authors

Author contributions to the practical work:

Francesca Scolari◐:	Half of the experiments (with focus on fitness tests), and partial*
Marc F. Schetelig◐:	Half of the experiments (with focus on molecular analysis), besides*
Sabrina Bertin:	*Sperm count analysis

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RESEARCH PAPER

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Research Paper

Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae)

Francesca Scolari[1,3], Marc F. Schetelig[2,3], Sabrina Bertin[1], Anna R. Malacrida[1], Giuliano Gasperi[1] and Ernst A. Wimmer[2]

[1] Dipartimento di Biologia Animale, Università di Pavia, Piazza Botta 9, 27100 Pavia, Italy
[2] Department of Developmental Biology, Göttingen Center for Molecular Biosciences, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, Georg-August-University Göttingen, GZMB, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany

The Sterile Insect Technique (SIT) involving area-wide release of mass-reared and sterilized pest insects has proven successful to reduce, control and eradicate economically important pest species, such as the Mediterranean fruit fly (medfly). For the efficient application, effective monitoring to assess the number and mating success of the released medflies is essential. Here, we report sperm-specific marking systems based on the spermatogenesis-specific *Ceratitis capitata β2-tubulin* (*Ccβ2t*) promoter. Fluorescent sperm can be isolated from testes or spermathecae. The marking does not cause general disadvantages in preliminary laboratory competitiveness assays. Therefore, transgenic sperm marking could serve as a major improvement for monitoring medfly SIT programs. The use of such harmless transgenic markers will serve as an ideal initial condition to transfer insect transgenesis technology from the laboratory to field applications. Moreover, effective and easily recognizable sperm marking will make novel studies possible on medfly reproductive biology which will help to further improve SIT programs.

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae), is one of the most devastating and economically important insects [1]. A most effective and environmentally safe approach to the species-specific control of this pest is the Sterile Insect Technique (SIT) [2], which reduces a pest population by mass release of reproductively sterile male insects into a wild-type (WT) population of the same species. This leads to the decrease of progeny by the competition of radiation-sterilized males with WT males for WT females [3]. In SIT programs besides the mass rearing, sterilization and releasing of the pest species, monitoring is of major importance. To directly monitor the relative size of wild populations and to calculate the ratio of released to wild insects, data from field traps are used. Currently mass-reared pupae are sterilized and dusted with fluorescent dyes for field monitoring in medfly SIT programs [4]. Thus, sterilized flies can be distinguished from WT flies when recaptured in traps within the release area. However, this marking procedure for monitoring implies several disadvantages: the fluorescent dyes are expensive, dangerous for human health and error prone [5]. Morphological markers could be alternatives to dusted flies, but they are often associated with a loss of overall competitiveness [6]. However, flies dusted with fluorescent dyes or carrying morphological markers cannot be used to get data on the mating status of WT females, which is important to assess the efficacy of SIT programs. Whether a WT female has mated to a WT male, a released sterile male or perhaps to both, can be checked by analyzing the progeny of live-trapped females [7] or by comparing the sperm head lengths of sperm in the spermathecae of mated females [8]. Sterilized sperm heads are shorter than WT ones [9]. This size differentiation despite being widely applied (Beatriz Jordão Paranhos and Donald O. McInnis, personal communication) is cumbersome and methods such as analyzing the progeny of live-trapped females are even more laborious.

For monitoring, transgenetically marked sperm can serve as an excellent alternative. Such marking systems have been described for the mosquitoes *Anopheles stephensi* [10] and *Aedes aegypti* [11]. In both systems a spermatogenesis-specific *β2-tubulin* (*β2t*)

Corresponding author: Wimmer, E.A. (ewimmer@gwdg.de)
[3] These authors contributed equally.

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New Biotechnology • Volume 25, Number 1 • June 2008 RESEARCH PAPER

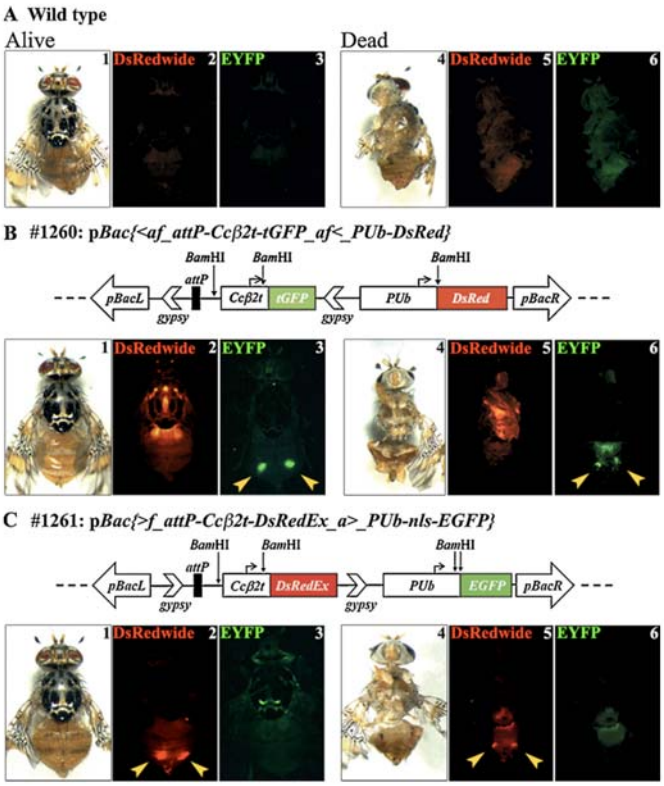

FIGURE 1

Sex-specific marked alive and dead males. Both alive (A1–3, B1–3, C1–3; dorsal view) and dead (A4–6, B4–6, C4–6; ventral view, three months after death) males were observed with different filter sets: cold light source, DsRedwide filter and EYFP filter. **(A)** Alive and dead WT males show no or weak autofluorescence with the EYFP and DsRedwide filter. **(B)** Schematic representation of the transgene #1260: p*Bac{<af_attP-Cβ2t-tGFP_af<_PUb-DsRed}* (not to scale). Alive (B2) and dead (B5) males show red fluorescence in the head, thorax and abdomen. Testes show strong green fluorescence (B3 and B6; yellow arrowheads). In dead males both markers are stable for at least three months (B5 and B6). Images were taken on males from line #1260_F-1_m-2. **(C)** Schematic representation of the transgene #1261: p*Bac{>f_attP-Cβ2t-DsRedEx_a>_PUb-nls-EGFP}* (not to scale). The transgene contains the *Cβ2t* promoter fused to *DsRedEx* and a *PUb*-driven EGFP [16]. Ventral head (not shown) and dorsal thorax (C3) show green fluorescence. Testes (C2 and C5; yellow arrowheads) are strong red fluorescent. The red testes marker is stable for at least three months in the dead males (C5). EGFP fluorescence is not visible in dead males (C6). Images were taken on males from line #1261_F-5_m-4. A 52-bp *attP* site is indicated as black bar; white arrowheads represent 0.4-kb *gypsy* fragments (5′–3′ orientation). *Bam*HI restriction sites are shown by vertical arrows.

promoter from *Anopheles gambiae* or *Aedes aegypti* drives testes-specific expression of a fluorescent marker. These systems thus combine inheritable fluorescence with sex-specific marking of the male testes. Here, we report the development of two testes-specific markers for medfly using the newly isolated spermatogenesis-specific medfly *β2t* promoter driving the expression of a red or green fluorescent protein. These markers will make improved monitoring possible for SIT programs and enable novel studies on medfly reproductive biology related to the mating behavior of this polyandrous species, such as sperm transfer, sperm storage, sperm use, sperm precedence and sperm competition [12,13].

Results

Isolation of medfly β2-tubulin promoter and generation of testes-specific marker constructs

To generate a sperm-specific marker system in the medfly, we isolated the *Cβ2t* (Genbank accession number EU386342) by degenerate primer PCR, RACE and inverse PCR. We fused the *Cβ2t* promoter (Genbank accession number EU386341) to the genes of the 'fast-folding' fluorescent proteins turboGFP (tGFP [14]) and DsRedExpress (DsRedEx [15]) to generate sperm-specific markers. These were then used to engineer four constructs based on *piggyBac* vectors carrying polyubiquitin (PUb)-driven EGFP or DsRed germline transformation markers [16,17]: p*Bac{fa-f_attP-Cβ2t-tGFP_a_PUb-DsRed}* (#1258), p*Bac{f_attP-Cβ2t-DsRe-dEx_a_PUbnlsEGFP}* (#1259), p*Bac{<af_attP-Cβ2t-tGFP_af<_PUb-DsRed}* (#1260) and p*Bac{>f_attP-Cβ2t-DsRedEx_a>_PUbnlsEGFP}* (#1261). All four constructs carry a minimal *attachment P* (*attP*) site [18], which will enable site-specific integration to potentially modify the transgenic situation. Moreover in the transformation vectors #1260 and #1261 the sperm-specific marker was flanked by *gypsy* insulator elements (> = *gypsy* element in 5′–3′ orientation; Fig. 1), which should reduce varieties in expression strength of the sperm-specific markers owing to position effects mediated

RESEARCH PAPER New Biotechnology • Volume 25, Number 1 • June 2008

by different genomic integration sites. These four vectors were used to germline transform medfly. The vector #1260 (Fig. 1B) was injected into 445 embryos of which 120 larvae hatched and 23 survived to adulthood (11 G0 females and 12 G0 males). Four female crossings (2–3 G0 females crossed to 5 WT males; F-1–F-4) and 4 male crossings (3 G0 males crossed to 15 WT females; M-1–M-4) were set up. G1 adults were screened for tGFP and DsRed fluorescence. Three of the female crossings (F-1, F-2 and F-3) and 1 of the male crossings (M-3) had fluorescent progeny with different red patterns and all transgenic males showed testes-specific tGFP expression. Thirteen male or female flies with different DsRed expression patterns (2 for F-1, 8 for F-2, 2 for F-3 and 1 for M-3) were backcrossed twice to WT to recognize possible multi-insertions by different PUb–DsRed patterns. The vector #1261 (Fig. 1C) was injected into 376 embryos of which 85 larvae hatched and 14 survived to adulthood (10 G0 females and 4 G0 males). Five female crossings (2 G0 females crossed to 5 WT males; F-1–F-5) and 4 male crossings (1 G0 male crossed to 6–15 WT females; M-1–M-4) were set up. G1 adults were screened for DsRedEx and EGFP fluorescence. Two of the female crossings (F-2 and F-5) had fluorescent progeny showing different green fluorescent expression patterns with all transgenic males having testes-specific DsRedEx expression. Eight male or female flies with different EGFP expression patterns (6 for F-2 and 2 for F-5) were backcrossed twice to WT to recognize potential multi-insertions by different PUb–EGFP patterns. For injections with *piggyBac* vectors #1258 and #1259, lacking the flanking *gypsy* insulators, no transgenic flies could be identified, despite a similar number of injected embryos, larval hatchings, adult survival and fecundity (data not shown).

Sex- and tissue-specific marker expression

Males transformed with the construct #1260 expressed *Cc*β*2t* promoter-driven tGFP in the testes (Fig. 1B3). The PUb–DsRed marker varied in the red fluorescence pattern between the different lines as previously described for PUb–DsRed [19], whereas the pattern itself was stable for each independent line. Both body and testes markers are highly stable in alive flies (Fig. 1B2 and 3) as well as in dead flies three months after death (Fig. 1B5 and 6). By contrast, alive or dead WT flies show no or only weak autofluorescence (Fig. 1A1–6).

Males transformed with the construct #1261 expressed *Cc*β*2t* promoter-driven DsRedEx in the testes (Fig. 1C2 and 5). The green fluorescence pattern varied because of position effects of the PUb–EGFP marker in different lines. The testes-specific DsRedEx expression was stably detectable in dead males three months after death (Fig. 1C5), whereas the green EGFP fluorescence lost intensity two to three days after eclosion in alive flies (data not shown) and was not detectable in dead flies (Fig. 1C6).

Strategy for generating homozygous lines

To verify transgenic lines (preliminarily screened for homozygous condition by fluorescence patterns and intensity) as genetically homozygous, we followed a strategy including three molecular techniques. First, Southern blots to *Bam*HI-digested genomic DNA of the lines #1260_F-1_m-2 and #1260_F-3_m-1 using a *DsRed*-specific probe (Fig. 2A) and of the lines #1261_F-5_m-4 and #1261_F-5_m-5 using a *EGFP*-specific probe (Fig. 2B) indicated single copy integration of the respective constructs. Second, 5′ and 3′ insertion site sequences were isolated by inverse PCR and verified correct *piggyBac*-mediated integration at canonical *TTAA* target sites (see Supplementary Methods). Third, characterization of flies by multiplex PCR [20] was carried out to verify and establish homozygous lines. For this, we designed two specific primers to the *piggyBac* ends and one primer for each flanking sequence (Fig. 2C diagram). From ten single crossings of putative homozygous flies, egg collections were taken separately. Thereafter a multiplex PCR was performed separately on genomic DNA from each single parent. Therefore, we were able to identify each parent molecularly as a homozygous or heterozygous individual for the lines #1260_F-1_m-2, #1260_F-3_m-1 and #1261_F-5_m-5. The progeny of all the single couples in which both parents were molecularly proven homozygotes were grouped to reduce bottleneck effects in the gene pool. In line #1261_F-5_m-4, the transgene integrated into a *mariner*-like transposon. Therefore, the multiplex PCR could not be used to identify homozygous situations and this line was checked for homozygous condition by more time-consuming test crosses to WT.

Mating ability of transgenic testes-marked medflies

To assess whether the testes-marked homozygous medfly lines show any major biological fitness problem, we tested their ability to mate and sire progeny in preliminary laboratory competitiveness tests. Transgenic males were tested at a 1:1:1 ratio competing with WT males for copulation with WT females (Table 1). *G*-test analyses showed that the ten replicates run for each line were statistically homogenous (#1260_F-3_m-1: $G = 4.7$. #1260_F-1_m-2: $G = 1.6$. #1261_F-5_m-5: $G = 4.2$. #1261_F-5_m-4: $G = 3.0$. df = 9 and $P > 0.05$ for all lines). #1260_F-3_m-1 males made 47% of the total matings and a *G*-test on the total mating number resulted in a nonsignificant difference between WT and transgenic performance ($G = 0.5$; df = 1; $P > 0.05$), which indicates the absence of a negative load for transgenic males in obtaining copulations. However, transgenic males from the other three transgenic lines had significant less matings compared to WT (Table 1; #1260_F-1_m-2: $G = 18.4$. #1261_F-5_m-5: $G = 81.7$. #1261_F-5_m-4: $G = 102.5$. df = 1 and $P < 0.001$ for all three lines). The results obtained from control cages in which only WT males were present showed that the total mating frequency was independent from the composition of the experimental cage and a *G*-test ($G = 5.91$; df = 4; $P > 0.05$) indicated that the presence of transgenic males did not disturb the mating tendency of the females.

Moreover, we compared the copulation latency [21] of transgenic and WT males and observed that transgenic males were not slower than WT in gaining copulation (#1260_F-3_m-1 versus WT: $t = 0.3$, df = 175. #1260_F-1_m-2 versus WT: $t = 0.8$, df = 184. #1261_F-5_m-5 versus WT: $t = 1.2$, df = 163. #1261_F-5_m-4 versus WT: $t = 0.1$, df = 166. $P > 0.05$ for all lines).

Adult longevity and paternity success

As an additional assessment of the overall fitness of the testes-marked homozygous medfly lines, we compared the longevity of WT and transgenic flies. Adult longevity of both #1260_F-3_m-1 and #1260_F-1_m-2 males was nonsignificantly different from WT as shown by *t*-tests (#1260_F-3_m-1 versus WT: $t = 0.23$. #1260_F-1_m-2 versus WT: $t = 0.23$. df = 48 and $P > 0.05$ for both lines). On the contrary, #1261_F-5_m-5 and #1261_F-5_m-4 males showed a

New Biotechnology • Volume 25, Number 1 • June 2008 RESEARCH PAPER

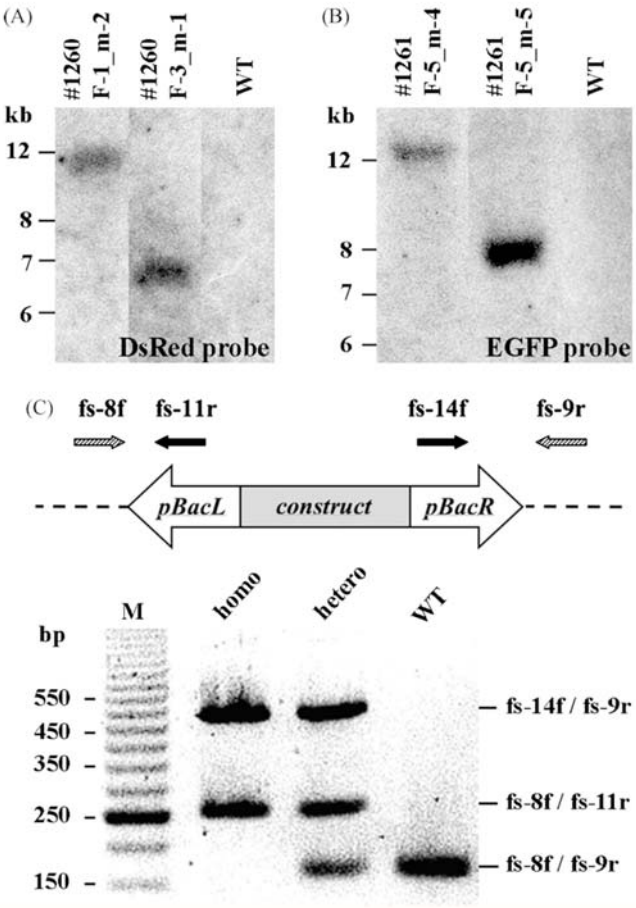

FIGURE 2

Strategy for generating homozygous transgenic lines. *Bam*HI-digested genomic DNAs isolated from indicated medfly lines were hybridized by using a DsRed **(A)** or an EGFP **(B)** probe. WT genomic DNA was used as a control for both. A single band in each lane indicates unique integration of the transgenes. **(C)** A strategy for generating homozygous lines is shown for #1260_F-3_m-1 as an example. Eggs from ten single mating pairs were collected separately. Subsequently, genomic DNA was isolated from each single parent fly and used as template for independent multiplex PCR reactions with two primers specific to the *piggyBac* ends (black arrows) and two primers depending on the integration site sequence as derived by inverse PCR (hatched arrows); specific *piggyBac* primers have been designed for individual lines to minimize interference with the integration site-specific primers. In homozygous situation (homo) two bands of 474 bp (amplified by fs-14f/fs-9r) and 254 bp (amplified by fs-8f/fs-11r) are expected, while in the heterozygous individuals (hetero) an additional band of 166 bp (amplified by fs-8f/fs-9r) is expected, which corresponds to the WT genomic situation. The progeny of the single mating pairs in which both parents were homozygous were pooled together and maintained as homozygous line. M: 50 bp ladder.

significantly shorter survival (#1261_F-5_m-5 versus WT: $t = 2.58$. #1261_F-5_m-4 versus WT: $t = 2.15$. df = 48 and $P < 0.05$ for both lines) (Fig. 3A). Nonsignificant differences in female adult longevity were observed between WT and transgenic lines (#1260_F-3_m-1 versus WT: $t = 1.83$. #1260_F-1_m-2 versus WT: $t = 1.11$. #1261_F-5_m-5 versus WT: $t = 0.48$. #1261_F-5_m-4 versus WT: $t = 1.75$. df = 48 and $P > 0.05$ for all lines) (Fig. 3B).

Furthermore, to analyze the effect of transgenic marking on the reproductive capacity, we compared the proportion of progeny sired by transgenic or WT males in a laboratory competition assay (Fig. 3C–F). In two experimental replications, males from lines #1261_F-5_m-5 and #1261_F-5_m-4 did show reduced competitiveness in these tests. However, males from lines #1260_F-3_m-1 and #1260_F-1_m-2 demonstrated to have great success in siring progeny in competition to WT males, which indicates that the transgenic marking does not generally cause a reduction in competitiveness.

Sperm-specific expression of fluorescent markers

To see whether the *Cc*β*2t* promoter-driven fluorescence could serve to follow individual sperm, we analyzed dissected testes and spermathecae in detail (Fig. 4). The testes-limited fluorescence in medfly (Fig. 1) confirmed the spermatogenesis-specific expression of *β2t* [22] and showed that the isolated 868-bp 5′ region

RESEARCH PAPER New Biotechnology • Volume 25, Number 1 • June 2008

TABLE 1

Mating ability of transgenic males competing with WT males

Transgenic lines	♀ mated to transgenic ♂ (tot matings)
#1260_F-3_m-1	47% (177)^{NS}
#1260_F-1_m-2	34% (186)***
#1261_F-5_m-5	16% (165)***
#1261_F-5_m-4	13% (168)***

Relative mating success of transgenic males in competition with WT males for copulations with WT females. For each transgenic line, 25 males were maintained in a cage with 25 WT females and 25 WT males and the number of total matings was obtained in 10 experimental replications. The percentage of matings attributed to transgenic males is reported. *G*-tests were used to reveal significant differences in mating number between transgenic and WT males (df = 1 in all the *G*-tests; significance levels: ***=$P < 0.001$; NS = nonsignificant).

contains the regulatory elements responsible for sex- and tissue-specific expression. Fluorescent spermatid bundles were isolated from males of lines #1260_F-3_m-1, #1260_F-1_m-2 (Fig. 4B3), #1261_F-5_m-5 and #1261_F-5_m-4 (Fig. 4C2). Dissected spermathecae from WT females mated to transgenic males from #1260_F-3_m-1 or #1260_F-1_m-2 (Fig. 4B6) and #1261_F-5_m-5 or #1261_F-5_m-4 (Fig. 4C5) carried respective green or red fluorescent sperm and additional fluorescent material transferred during mating. By contrast, WT females mated to WT males did not show such red or green fluorescent materials (Fig. 4A5 and 6) and single sperm separated from such spermathecae showed neither red nor green autofluorescence (Fig. 4A8 and 9). Single sperm derived from transgenic males showed the respective green or red fluorescence restricted to the sperm tail. Sperm of #1260_F-3_m-1 showed in addition to green also red fluorescence, which is most probably due to a position effect on the PUb–DsRed marker (data not shown). The detectability of fluorescently marked sperm in spermathecae and the results that marked sperm does not in principle interfere with mating and paternity success indicate the potential use of such a marker for monitoring purposes in medfly SIT programs.

Studying reproductive biology

Furthermore, the ability to easily detect marked sperm after the transfer from males to females enables effective studies of reproductive biology, such as sperm transfer and sperm use. To demonstrate this, we compared the number of transgenic or WT sperm stored by WT females after successful copulation (Table 2). The number of females with empty spermathecae [23] resulted to be statistically heterogeneous across the lines ($G = 25.75$; df = 4; $P < 0.001$). This was because of both a significant difference within the transgenic lines ($G = 15.51$; df = 3; $P < 0.001$) and between transgenic (pooled together) and WT ($G = 10.23$; df = 1; $P < 0.001$). Successful sperm transfer appeared particularly compromised for #1261_F-5_m-5 males.

Sperm count data were pooled from two replicates covering two generations and statistically tested for homogeneity (WT: $t = 0.44$; df = 48. #1260_F-3_m-1: $t = 1.37$; df = 59. #1260_F-1_m-2: $t = 1.25$; df = 70. #1261_F-5_m-5: $t = 1.34$; df = 52. #1261_F-5_m-4: $t = 1.69$; df = 40. $P > 0.05$ for all lines). ANOVA across the four transgenic lines did not result in any significant difference ($F = 1.46$; df = 3, 131; $P > 0.05$). However, ANOVA showed that WT and transgenic lines were statistically heterogeneous ($F = 3.93$; df = 4, 171; $P < 0.01$). The comparison between transgenic lines (pooled together) and WT showed a high significance ($F = 11.41$; df = 1, 171; $P < 0.001$), demonstrating that the number of sperm released by transgenic males was significantly lower than the number transferred by WT.

In an identical but independent experimental set-up, we examined the time line of WT or transgenic sperm use (Fig. 5). A *G*-test indicates that larval hatch-rates declined over time for WT as well as all the transgenic lines (WT: $G = 136.6$. #1260_F-3_m-1: $G = 542.7$. #1260_F-1_m-2: $G = 712.2$. #1261_F-5_m-5: $G = 958.3$. #1261_F-5_m-4: $G = 760.3$. df = 2 and $P < 0.001$ for all). At each egg collection

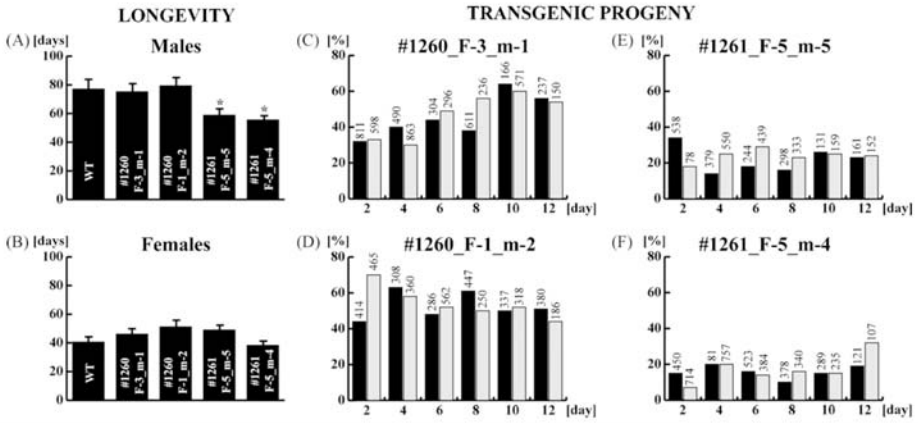

FIGURE 3

Adult longevity and paternity success of the transgenic flies. Mean survival of WT and transgenic males **(A)** and females **(B)**. Bars represent average number (±SE) of survival days. Twenty-five flies were monitored for each line. *, transgenic lines with significantly shorter lifespan than WT. **(C–F)** For each of the four indicated transgenic lines, 25 males were maintained in a cage with 25 WT females and 25 WT males to determine the relative success in siring progeny of transgenic males competing with WT males. Eggs laid within 24 hours were collected every two days for two weeks starting three days after eclosion. The experiment was done twice for each transgenic line (black and gray bars). The percentage of progeny attributed to transgenic males is shown with total number of flies on top of the bars.

New Biotechnology • Volume 25, Number 1 • June 2008 RESEARCH PAPER

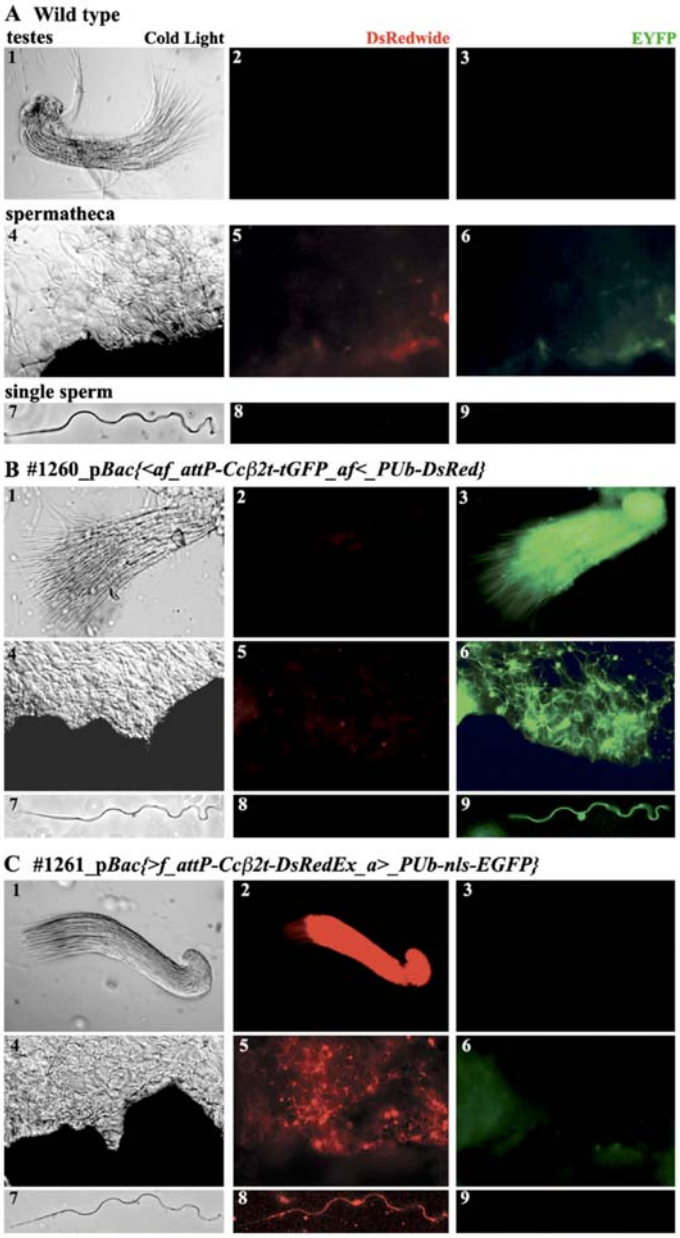

FIGURE 4

Fluorescence in testes, spermathecae and single sperm. Spermatid bundles dissected from male testes (1–3), mechanically opened spermathecae from WT females mated to WT or transgenic males (4–6) and single sperm from these spermathecae (7–9) are depicted with phase contrast or epifluorescence with the Zeiss filters sets 13 and 20. **(A)** The WT spermatid bundle (A1–3) and single sperm (A7–9) show no red or green autofluorescence. The intact WT spermatheca is dark black (not shown) and when broken open shows weakly red and green autofluorescenting material (A4–6). **(B)** The spermatid bundle and the sperm from the spermatheca (female mated to a male of line #1260_F-1_m-2) show strong green fluorescence (B3, 6 and 9). Additional green fluorescent material is detected along the sperm and in the spermatheca (B9 and 6). **(C)** The spermatid bundle and the sperm from the spermatheca (female mated to a male of line #1261_F-5_m-4) show red fluorescence (C2, 5 and 8). Additional strongly red fluorescent material is visible along the sperm and in the spermatheca (C8 and 5).

TABLE 2

Average number of mating-transferred sperm

Medfly lines	% ♀ with sperm (*n* tot)	Average sperm number ± SE
WT	82 (50)	2364 ± 217
#1260_F-3_m-1	64 (61)	1854 ± 232
#1260_F-1_m-2	71 (72)	1625 ± 174
#1261_F-5_m-5	27 (54)	1164 ± 176
#1261_F-5_m-4	60 (42)	1532 ± 198

Percentage of females that had a mating with actual sperm transfer is indicated and the sample size (number of mating engaged and dissected females) is shown in parenthesis. Average number (±SE) of sperm counted from dissected spermathecae of WT females mated to WT or transgenic males. The average sperm number is calculated on the total number of samples, excluding the females whose mechanically broken spermathecae were found empty.

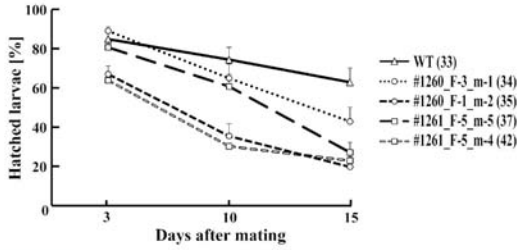

FIGURE 5

Time course of larval hatching. The hatching rate was determined on egg collections of three time points: 3, 10 and 15 days after mating between WT females and either WT or transgenic males had occurred. Each point represents the mean proportion (±SE) of hatched embryos on total eggs laid within 24 hours. The respective sample size (number of females scored) is indicated in the inlet legend.

time point (3, 10 and 15 days after the mating), the proportion of hatched eggs was statistically heterogeneous across the lines (df = 4; $P < 0.001$ in all the *G*-tests). This was because of both a significant difference within the transgenic lines (df = 3; $P < 0.001$ in all the *G*-tests) and between transgenic (pooled together) and WT (df = 1; $P < 0.001$ in all the *G*-tests). On the one hand, this suggests that transgenic sperm was not as efficiently used over time as WT sperm, but on the other hand, this also indicates that transgenic sperm was still used 15 days after the mating.

Discussion

With this study we provide a powerful sperm-marking system for medfly. Our aim was to obtain direct sperm visualization, both for applied and basic purposes: first, to provide an efficient tool for improving the monitoring of ongoing SIT programs and second, to improve the analysis of medfly mating physiology regarding sperm transfer, storage, competition and use. We show that the endogenous *β2t* promoter could be used in the medfly to generate fluorescently labeled sperm as was previously described for mosquitoes [10,11]. The fluorescent marking makes it possible to separate males from females and to clearly identify the testes as well as single sperm at adult stages. In contrast to the mosquito studies, however, the fluorescence in the testes is only sporadically detectable at the last larval stage despite the use of fast-folding fluorescent proteins, which is therefore not suitable to create larval sexing and sex-separation techniques in medfly. For the clean and rapid establishment of homozygous transgenic lines – which are necessary for assays on sperm competition – we developed a molecular method based on multiplex PCR that can be applied to nonmodel organisms. When the homozygous transgenic lines were compared to WT in different preliminary laboratory competitiveness tests, several lines showed reduced fitness indicating that the genetic bottleneck during the lines' establishment, the presence of the transgene, or its particular insertion into the genome can cause fitness costs [24]. In spite of this, males of one sperm-marked line, #1260_F-3_m-1, showed no significant reduction in their overall fitness transmitting their genes to the next generation in a competitive way. This shows the advantage of creating many independent transgenic lines to choose competitive ones. Actually, our constructs introduced *attP* sequences, which will make it possible to use the site-specific integrase system from phage *phiC31* [25] to add additional functional transgenes into this fit sperm-marked line #1260_F-3_m-1 at the same genomic position [26]. Moreover, line #1260_F-3_m-1 shows both red and green fluorescence in the sperm – probably on account of a position effect on the PUb-driven DsRed body marker – that indicates that the strong expression of fluorescent proteins is not harmful to the sperm's function. The laboratory competitiveness tests performed here can only be a first step into the evaluation of the fitness and stability of such transgenic lines, which need to be followed by mass rearing tests and field cage evaluation by setting competition between transgenic and endemic, locally sampled, WT flies [27]. A simple distinction between WT sperm and sperm from released sterile males will greatly improve monitoring SIT programs, which is a key component to run this environment-friendly pest management successful and cost effective. The combination of the presented fluorescent markers could serve excellently for this purpose. Owing to the stability of the used fluorescent proteins [28], both the red fluorescence (DsRed) in the body as well as the green fluorescence in the testes (tGFP) are stable several months after death. Therefore, even dead flies captured in traps within the SIT release area could be scored to calculate the ratio of released to wild insects. In addition, the sperm marking would make it possible to more easily assess the mating status of trapped females, which would allow for controlling the mating efficiency of the released sterile males.

The high quality control during the production of sterile insects for SIT as well as the fact that sterilized insects are incapable of passing their genes on to the next generations provide ideal initial conditions for first releases of genetically engineered insects into the field [29,30]. Before fertile transgenic insects can be released in economic and medical field applications, ecological risk assessments are necessary. Nevertheless, to assess such potential risks, at some point transgenic insects will have to be released. For this purpose, SIT programs that use sterilized transgenic insects carrying harmless, fluorescent-protein-based markers – as described in this study – will be most appropriate. Owing to the sterility, exposure of the environment to the transgenes will be limited to the lifespan of the released individuals. Transgene constructs containing only fluorescent protein markers will be most suitable to transfer transgenic technology from the laboratory to the field, since they will improve SIT applications by simplifying the monitoring, should not provide advantages to the carrier organism and should allow the

identification of carriers at later stages. Such initial transgenic SIT programs could then provide the first ecological insights into the fitness of transgenic insects and their distribution [31]. Furthermore, the availability of homozygous medfly lines with differently marked sperm (tGFP or DsRedEx) will enable novel studies on the reproductive biology of this polyandrous species. Thus old and new questions on mating physiology, cryptic female choice, sperm competition, sperm quality and other postcopulatory sexual behaviors can now be addressed [32]. And in turn, an enhanced knowledge of these basic biological processes will provide additional means to further improve environmentally safe, biological pest management approaches.

Materials and methods

Details on inverse PCR, cloning strategy, primers, germline transformation, imaging, Southern blot analyses and fitness tests are shown in Supplementary Methods.

Medfly samples

WT and transgenic medfly lines were maintained under standard rearing conditions [33]. The WT strain *Egypt-II* was obtained from the FAO/IAEA Agriculture and Biotechnology Laboratory (Seibersdorf, Vienna, Austria). The fitness tests were performed on homozygous transgenic lines maintained in the lab for six to ten generations.

Isolation of medfly β2-tubulin

On the basis of protein alignments of *β2-tubulin* genes from *Drosophila melanogaster*, *Drosophila hydei*, *Anopheles gambiae*, *Danio rerio*, *Gallus gallus* and *Homo sapiens*, degenerate primers were designed to possibly exclude or at least minimize the isolation of *β1-* and *β3-tubulin* sequences. To isolate polyA$^+$ mRNA from testes, we dissected 36 males, put the testes into RNAlater solution (Ambion, Austin) and extracted polyA$^+$ RNA using Micro Poly(A) Pure (Ambion, Austin). Three cDNA pools were generated using this testes-specific mRNA: (i) a double-strand cDNA using BD SMART PCR cDNA Synthesis Kit (BD Biosciences, Heidelberg); (ii) a 5′ single-strand cDNA for RACE and (iii) a 3′ single-strand cDNA for RACE using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Heidelberg). A 523-bp fragment of the *β2-tubulin* was isolated with degenerate primers mfs-100 and mfs-102 on the double-strand cDNA pool (1.5 min at 94 °C; 31 cycles of 30 s at 94 °C, 1 min at 50 °C, 1.5 min at 68 °C; and 5 min at 68 °C). To amplify the 5′- and 3′-UTR of *β2-tubulin* we did RACE PCR on a 5′ and a 3′ single-strand cDNA pool using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Heidelberg) and the gene-specific primers mfs-150 for the 5′-end and mfs-155 for the 3′-end. The genomic upstream region of *Ccβ2t* was then isolated by inverse PCR, sequenced and finally an 868-bp 5′ region including the *Ccβ2t* promoter was amplified by PCR from genomic DNA (Supplementary Fig. 1).

Multiplex single-fly PCR

To identify homozygous transgenic individuals, genomic DNA of single flies was isolated as described [34]. Five microliters of the single-fly homogenates were used for multiplex PCR reactions (2 min at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C; and 5 min at 72 °C) using BD Advantage 2 PCR (BD Biosciences, Heidelberg). Each multiplex primer mix contained four primers: a WT-specific forward primer (primer fs-15f for line #1260_F-1_m-2, fs-8f for #1260_F-3_m-1 and fs-27f for #1261_F-5_m-5), a WT-specific reverse primer (primer fs-16r for line #1260_F-1_m-2, fs-9r for #1260_F-3_m-1 and fs-28r for #1261_F-5_m-5), a *piggyBac*-specific forward primer (primer fs-20f for line #1260_F-1_m-2, fs-14f for #1260_F-3_m-1 and fs-30f for #1261_F-5_m-5) and a *piggyBac*-specific reverse primer (primer fs-18r for line #1260_F-1_m-2, fs-11r for #1260_F-3_m-1 and fs-29r for #1261_F-5_m-5). Each PCR reaction was analyzed on a 2.0% agarose gel for the presence or absence of WT and transgene insertion-specific bands.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbt.2008.02.001.

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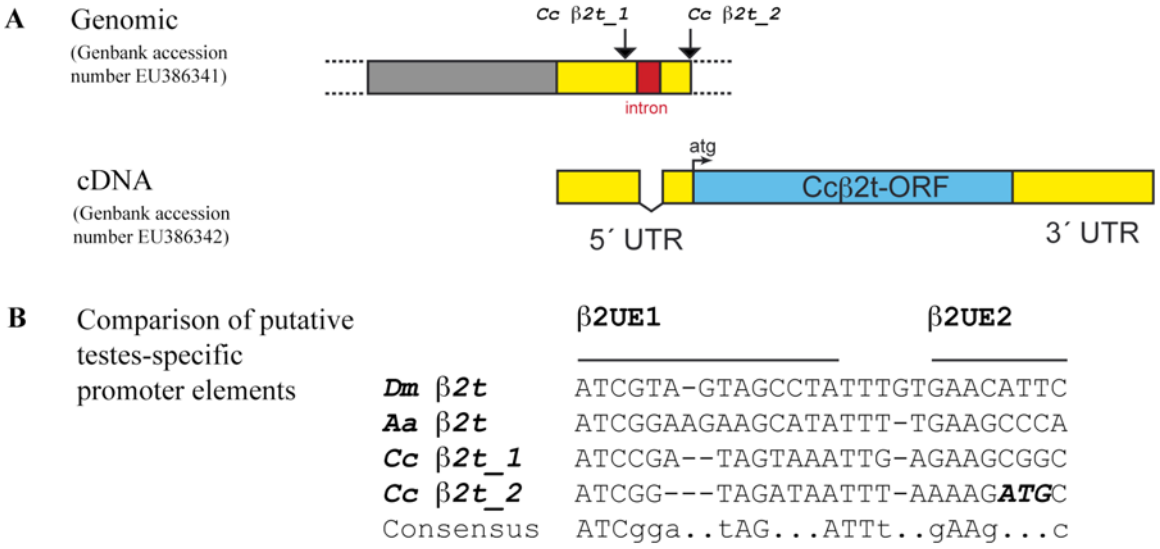
Supplementary



Supplementary Figure 1. Partial gene structure and putative promoter elements of medfly *β2-tubulin*

(A) A schematic diagram of the medfly *β2-tubulin* upstream region as well as 5´UTR isolated from genomic DNA (not to scale; Genbank accession number EU386341) and the medfly *β2-tubulin* cDNA (1859 bp; not to scale; Genbank accession number EU386342) are shown. The putative promoter region of 544 bp (grey) is followed by the 384 bp 5´ UTR (yellow), which contains a 79 bp intron starting at position +221 (red). The two putative testes-specific promoter elements *Cc β2t_1* and *Cc β2t_2* in the 5´UTR are indicated. The 1338 bp ORF flanked by a 5´ (305 bp) and a 3´ (214 bp) UTR (yellow) is shown in blue.

(B) Sequence alignment of putative *β2-tubulin* promoter motifs. A sequence from *Drosophila melanogaster*, containing the spermatocyte-specific β2UE1 and the quantitative regulating β2UE2 motif[1], a sequence from *Aedes aegypti*[2] and the two *in silico* identified putative promoter motifs from medfly (*Cc β2t_1* and *Cc β2t_2*) were aligned.

Supplementary Methods

Inverse PCR

To get the 5´upstream region of *β2-tubulin*, inverse PCR was performed by digestion of 1.5 µg of medfly WT genomic DNA with *Mun*I. After 24 h digestion, restriction fragments were precipitated and self-ligated in a volume of 500 µl at 16°C for 24 h. PCR was performed on the circularized fragments by using primer sequences in opposite orientation within the medfly *β2-tubulin* 5´UTR. First, a PCR was performed with primers mfs-174 and mfs-176 (1 min at 95 °C; 6 cycles of 30 sec at 94 °C, 45 sec at 66 °C (-2°C each cycle), 5 min at 68 °C; 25 cycles of 30 sec at 94 °C, 45 sec at 54 °C, 5 min at 68 °C; and 6 min at 68°C) using BD Advantage 2 PCR (BD Biosciences, Heidelberg). Second, the obtained PCR product was diluted 1:250 with ddH_20 and a nested PCR with primers mfs-175 and mfs-177 was performed (1.5 min at 94 °C; 3 cycles of 30 sec at 94 °C, 30 sec at 60 °C (-3°C each cycle), 5 min at 68 °C; 20 cycles of 30 sec at 94 °C, 45 sec at 50 °C, 5 min at 68 °C; and 6 min at 68°C) using 5 µl of the dilution and the BD Advantage 2 PCR Kit (BD Biosciences, Heidelberg). An approximately 800 bp PCR product was cloned into a pCRII vector (Invitrogen, Karlsruhe) and sequenced (sequences in Supplementary Fig. 1 and Genbank accession number EU386341).

To localize the integration sites of *piggyBac* vectors, inverse PCR was performed with primers and protocols described[3]. Genomic DNA from the homozygous transgenic lines #1260_F-1_m-2, #1260_F-3_m-1, #1261_F-5_m-4 and #1261_F-5_m-5 was extracted as described[4], 1 µg digested by *Hae*III (line #1260_ F-3_m-1) or *Msp*I (lines #1260_F-1_ m-2, #1261_F-5_ m-4 and #1261_F-5_m-5) and circularized by self-ligation at 16°C in a volume of 500 µl for 24 h. PCR products (5 min at 95 °C; 5 cycles of 30 sec at 95 °C, 45 sec at 69 °C (-2°C each cycle), 3 min at 72 °C; 25 cycles of 30 sec at 94 °C, 45 sec at 59 °C, 3 min at 72 °C; and 7 min at 72°C) were cloned into pCR2.1-TOPO vectors (Invitrogen, Milan). Plasmids were sequenced with the standard primers M13 forward and M13 reverse. Sequence analysis was performed using Vector NTI 10 software (Invitrogen, Karlsruhe).

Sequences flanking *piggyBac* insertions. Sequences are shown in 5´ to 3´ orientation in respect to the *piggyBac* insertion. Duplicated integration sites (**ttaa**) indicated in bold, enzyme restriction sites underlined (GGCC = *Hae*III; CCGG = *Msp*I):

Line #1260_F-1_m-2

*CCGG*TCTCGAAAAAAAACATAATTAAAATGCATTTCAAATATTTTTTTTCTGAAACACCCTATTTTTTGAAATTA
CAACTTGAACGTTGATACTATATATGCATACGTGAATACTAAATATTTGAAATGCATTTAAAAAACGTTTATTTT
CGAAACCAGAAAGTGTATAACCCTGTACAAACAAACGCTAAGTATCGCTCATCTGCATTTTAT**TTAA...*piggy Bac*...TTAA**ACAGTTTCAGTCTAAAG*CCGG*

Line #1260_F-3_m-1

*GGCC*CACATTTGTCTAACTTCTGTTACGTGGTTCAGTAGTGAGTTTTGAGTAATAGCCTAAATGTAGATGT**TTAA
...*piggyBac*...TTAA**ACAAAAACAATTTTACTGCAATTATTGGAGAAAAAAATAATCATCTTTTAAGTTAGT
AGTTTTGAAGACAGAACTGAATACGCGTCCAATTCTAAAGCCGCGTTTCCACCAAT*GGCC*

Line #1261_F-5_m-4

*CCGG*CTCGAAAAAACGAGTCCTCTTTTGACGCTATCCACGAAGCAATCCATTTTTCCAATTCTTCGAAGGATTGA
AAATGTTGATCTGCCATACCGTGTGGTAGTCAGAAGGGGCGACATCTGGGGAATACGGCGGGTGGGGTAAGACTT
CCCATTTCAGCGTTTCCAGGTACTTTTTGACCACTTTTGCGACGTGAGGCCGAGCATTGTCGTGTTGGAGGCTGA
ATTTGTCATGTTGCTCTTGATATTGTGGCCGCTTTTCTTTTAGCGCGCCACTAAGGCGCATCAGTTGCGTTCGGT
AGCGATCTCCTGAGATGGTTCACCCGATTTTAAAAACTCGTAGTAAATCACACCGAGCTGATCCCACCAAATACA
AATCATAACCTTGGCGCCGTCAATATTCATTTTTAAAACGTTGAAACCACTCCCGACACGTTCTTTCACTCAGAG
CAGCATCACCGTAAGTTTCTGAGAGCATTCGATGCGCTTCAGCTGCATTTTTTTTTCGAATTGTAT**TTAA...*pi***
***ggyBac...*TTAA**CAGAAAAGTAAAACTTCCCGCAAATGGCGAGAATTAGGCACATAAACGGACATTTTCGAGCG
TGAATAATACGAAAACAAGAACAACTGTCACTGAAACGGCGATGACAATTCGTTAGGCACTGTCCACACTCACTT
TAAAGGCATTATCATCTATGTATTTCAACCAGCCTCAG*CCGG*

Line #1261_F-5_m-5

*CCGG*GCTTGGCAAAGAATATTCGACGCGTTTTTTGAAACAATGACAAAACCTTATCTTTATAGGAAAGCCAAATT
ATTGTAAACCAAAATGTAAGTGTAAAAACATTTCT**TTAA...*piggyBac...*TTAA**AGTTAAATTACAGTGGAG
GAGATAATAAAACTCGAGGTCAACTTCGGGCAGTTGGATAAATTGCAAGTGCGCATTGCACTTGGGTGATGTTGT
CCTCAGCAGGGGACATATGAGAAATGCGTGACGTTTACAGCTGAAAACTTGTACATTTCTCGTGGGGTACGCACT
GCCCGAAAGCCTACGTGATTAGCAGCAGCAATGCCATCTTTAAATAAGTATGCATATTTGTAAACAAAAAATCAG
ATGAAAAAGTTAATTTTAAGAAATTTATGTTTCGTTCATTTCATAAATATATATTTAATATTTTTTTTTTTTACA
ACAAGACAAGAAGTGTTAGTCGTTAATAATTCTTCAATTACAAAACATTTTAAATGAAAAGGTGAAGAAAAATGT
TTCTCTTGAGATATTTAAATAGAAATTGGATATTTCAACACAAAATCAATGATTAAAATGATTTGGAAAATGTGC
ATAGCAAGGTTTGTTTCCAAAACAACACTTCTCTGCTTGCGTATATGTATGTATGTATGTAGCATTTGCCTGTGA
ATGTTCGTACTTCAAGCAACGGTTAACATTTTAGACACGACGCAAAAAGCTGCAATCTGTGACTCAAGCAAACCA
AACACAATGCCAATGTATGTATGAATGTAGCACGCTTCCACCTCTACCTCACCTTAGCACATCCAACTATGCCAA
CGACAGCCAGCCAGCAGCTGTTTCGAAACTTG*CCGG*

Two-step cloning procedure

Generally we compose our constructs in the cloning shuttle vector pSLfa1180fa[5]. From the shuttle vectors the constructs can be easily placed in transformation vectors which carry *Fse*I and *Asc*I sites (*fa*-sites[5]).

Shuttle vectors

The pSL*af_attP-Ccβ2t-tGFP_af* with an *attP* site[6] of 52 bp was created by ligating annealed primers (mfs-201 and mfs-202) into the *Eco*RI cut pSL*af_Ccβ2t-tGFP_af*, which was created by ligating two fragments into the *Nde*I-*Hind*III cut pSLfa1180fa[5]: i) the *Ccβ2t* 5´ upstream region, isolated as a 868 bp *Nde*I-*Eco*31I fragment from pCRII-*Ccβ2t*; ii) a 922 bp fragment containing tGFP, isolated by PCR on pTurboGFP-PRL (Biocat, Heidelberg) with the primers mfs-184 and mfs-185 and cut with *Eco*31I-*Hind*III. The pSL*af_attP-Ccβ2t-DsRedEx_af* with an *attP* site of 52 bp was created by ligating annealed primers (mfs-201 and mfs-202) into the *Eco*RI cut pSL*af_Ccβ2t-DsRedEx_af*, which was created by ligating two fragments into the *Nde*I-*Hind*III opened pSLfa1180fa[5]: i) the *Ccβ2t* (868bp) upstream region, isolated as a *Nde*I-*Eco*31I fragment from pCRII-*Ccβ2t*; ii) a 899 bp fragment containing DsRedEx, isolated by PCR on pDsRedExpress-1 (Clontech, CA) with the primers mfs-186 and mfs-185, and cut with *Eco*31I-*Hind*III.

To yield pCRII-*Ccβ2t*, the 943 bp 5´ region ending at the ATG of the medfly *β2-tubulin* (Supplementary Fig. 1) was amplified by PCR on WT genomic DNA with primers mfs-209 and mfs-196, and ligated into the vector pCRII (Invitrogen, Karlsruhe).

Transformation vectors

p*Bac{fa_PUb-DsRed}* and p*Bac{af_PUb-DsRed}* or p*Bac{fa_PUbnlsEGFP}* and p*Bac{af_PUbnlsEGFP}* were generated by cloning hybridized primers mfs-117 and mfs-118 into the *Bgl*II site of pB[PUbDsRed1][7] or pB[PUbnlsEGFP][8], respectively. Two vectors with *gypsy* insulator flanked *fa*-sites were generated in two steps. In the following ">" symbolizes a 5´-3´ orientated *gypsy*[9] element. To yield p*Bac{<af<_PUb-DsRed}*, a 0.4 kb *gypsy* PCR fragment from p*Bac{3xP3-DsRed>af}*[10] amplified with the primers mfs-164 and mfs-166, cut with *Bst*XI and *Fse*I, was ligated into the *Fse*I cut p*Bac{<af_PUb-DsRed}*, which was created by ligating a 0.4 kb *gypsy* PCR fragment from p*Bac{3xP3-DsRed>af}* amplified with the primers mfs-165 and mfs-167, cut with *Asc*I and *Bgl*II, into the *Asc*I and *Bgl*II cut p*Bac{af_PUb-DsRed}*.

To yield p*Bac{>fa>_PUbnlsEGFP}*, a 0.4 kb *gypsy* PCR fragment from p*Bac{3xP3-DsRed>af}* amplified with the primers mfs-165 and mfs-167, cut with *Asc*I and *Bgl*II, was ligated into the *Asc*I and *Bgl*II cut p*Bac{>fa_PUbnlsEGFP}*, which was created by ligating a 0.4 kb *gypsy* PCR fragment from pBac*{3xP3-DsRed>af}* amplified with the primers mfs-164 and mfs-166, cut with *Bst*XI and *Fse*I, into the *Fse*I cut p*Bac{fa_PUbnlsEGFP}*.

#1258_p*Bac{faf_attP-Ccβ2t-tGFP_a_PUb-DsRed}* and *#1260*_p*Bac{<af_attP-Ccβ2t-tGFP_af<_PUb-DsRed}* were created by ligating the 2.1 kb *Asc*I fragment *af_attP-Ccβ2t-tGFP_a* from pSL*af_Ccβ2t-tGFP_af* into the *Asc*I site of p*Bac{fa_PUb-DsRed}* and p*Bac{<af<_PUb-DsRed}*, respectively. The #1259_p*Bac{f_attP-Ccβ2t-DsRedEx_a_PUbnlsEGFP}* and #1261_p*Bac{>f_attP-Ccβ2t-DsRedEx_a>_PUbnlsEGFP}* vectors were created by ligating the 2.0 kb *Fse*I-*Asc*I fragment *f_attP-Ccβ2t-DsRedEx_a* from pSL*af_attP-Ccβ2t-DsRedEx_af* into the *Fse*I-*Asc*I opened p*Bac{fa_PUbnlsEGFP}* and p*Bac{>fa>_PUbnlsEGFP}*, respectively.

Primer sequences

Sequences shown in 5´ to 3´ orientation.

mfs-100: GARGAGTAYCCIGAYMGIATHATG
mfs-102: GCATYTGYTCRTCIACYTCYTTCAT
mfs-117: GATCGGCCGGCCTTGGCGCGCCTA
mfs-118: GATCTAGGCGCGCCAAGGCCGGCC
mfs-150: TCGCCATAAGTGGGGGTAGTAAGTTTC
mfs-155: CGGGTTTCGCTCCACTGACTTCTCGT
mfs-164: GACTCCAGCCGGCTGGGGTACCCTATTCGCAAAAAC
mfs-165: CAGTGGCGCGCCGGTACCCTATTCGCAAAAAC

mfs-166: TGATGGCCGGCCTAGCTAAATGGTATGGCAAG
mfs-167: ACGGAGATCTGGCTAAATGGTATGGCAAG
mfs-174: AGTCGAAGATACTGTGAATTTCCATTC
mfs-175: CTGATCCTTGGATAAGTTATGCAATTTG
mfs-176: GAGAAGCGGCAAAACCTTAAGTAAAGTCC
mfs-177: CTCGTGCTATTCTGGTGGATTTAGAGC
mfs-184: TACTGGTCTCAATGGAGAGCGACGAGAGCGGCCTG
mfs-185: CTGTAAGCTTGACAAACCACAACTAGAATGCAGTG
mfs-186: AGCTGGTCTCAATGGCCTCCTCCGAGGACGTCATC
mfs-196: TCGAGGTCTCACCATCTTTTAAATTATCTCCCGATTTAATTACG
mfs-201: AATTCGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGGTC
mfs-202: AATTGACCCTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACG
mfs-209: CAACTTTTCAACGTCGAACCTTCCTTGTG
mfs-228:CCTCGATATACAGACCGATAAAACACATGCGTC
mfs-231:CAGTGACACTTACCGCATTGACAAGCACGC
1260DsRedf: GCTCCTCCAAGAACGTCATC
1260DsRedr: TGGTGTAGTCCTCGTTGTGG
1261EGFPf: GACGTAAACGGCCACAAGTT
1261EGFPr: GAACTCCAGCAGGACCATGT
fs-8f: GGCCCACATTTGTCTAACTTCT
fs-9r: AATTGGACGCGTATTCAGTTCT
fs-11r: CGCGGTCGTTATAGTTCAAAAT
fs-14f: TGTGCCAAAGTTGTTTCTGACT
fs-15f: CGCTAAGTATCGCTCATCTGC
fs-16r: CCGGCTTTAGACTGAAACTGT
fs-18r: CACGCGGTCGTTATAGTTCA
fs-20f: GTGCCAAAGTTGTTTCTGACTG
fs-27f: CAAAGAATATTCGACGCGTTTT
fs-28r: CATTTCTCATATGTCCCCTGCT
fs-29r: AAGAATGCATGCGTCAATTTTA
fs-30f: TGCCAAAGTTGTTTCTGACTGA

Germline transformation and depiction of transgenic tissue

Germline transformation experiments were performed by microinjection of *piggyBac* constructs (500 ng/µl) together with the p*hspBac* transposase helper plasmid[8] (200 ng/µl) into WT embryos as described by Handler[11] with the following exceptions: injected eggs were covered with Voltalef 10S oil (Lehmann & Voss, Hamburg), placed at 28°C in parafilm closed Petri dishes with watered Whatman paper in the lid; neither eggs, larvae nor pupae were heatshocked; eclosed G_0 males and virgin females were backcrossed in groups of 1-3 individuals to 5-15 virgin WT females or five WT males, respectively. G1 progeny were screened by epifluorescence for the expression of the *PUb-*

DsRed, PUb-EGFP, Ccβ2t-tGFP and *Ccβ2t-DsRedEx* using the fluorescence stereomicroscope Leica MZ16 FA with the filters DsRedwide (Ext. 546/12; Emm. 605/75) and EYFP (Ext. 500/20; Emm. 535/30). Images were taken with an Intas MP Focus 5000 digital camera. Sperm isolated from male testes and female spermathecae were screened under an epifluorescence Zeiss Axioplan microscope using the filters: Zeiss filter set 13 (Ext. 470/20; Emm. 505-530) for the screening of tGFP fluorescence and Zeiss filter set 20 (Ext. 546/12; Emm. 575-640) for the screening of DsRedEx fluorescence. Images were captured using an Olympus DP70 digital camera.

Southern hybridization

Genomic DNA (~3-10 µg) from adult flies of different transgenic lines and the WT strain were digested with *Bam*HI (Roche, Mannheim) and separated on 1% agarose gels. DNA was transferred to nylon filters and immobilized by UV irradiation. Probe labeling and membrane hybridizations were performed according to the AlkPhos Direct kit (GE Healthcare, Little Chalfont). Three 7 min post-hybridization washes were performed at 55°C. Further three 5 min washes were performed at room temperature. Signal detection was performed using CDP-star (GE Healthcare, Little Chalfont) followed by exposure for approximately 3 hours on Kodak X-Omat film.

The two probes for detecting DsRed (624 bp) or EGFP (609 bp) were amplified by PCR (2 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 58 °C, 1 min at 72 °C; 10 min at 72°C) from the constructs #1260 or #1261 with the primers 1260DsRedf and 1260DsRedr or 1261EGFPf and 1261EGFPr, respectively.

Test cross

The classical genetic approach of test cross was followed to check for homozygous condition of a single couple from the line #1261_F-5_m-4 tentatively recognized as homozygotes by strong fluorescence.

The single couple was allowed to mate and oviposed eggs were collected for ten days. Thereafter the male was isolated and backcrossed for six days to a virgin WT female. Oviposed eggs were collected every two days and reared to adults. All the progeny were screened by epifluorescence for the expression of the *PUb-EGFP* and had to be fluorescent to prove that the male was homozygous. To check for homozygous condition in the female, 20 single couples were set up from virgin progeny of the chosen parent couple. The oviposed eggs were separately collected and reared to adults. The progeny were then screened by epifluorescence. All the progeny of these 20 single couples have to be fluorescent to support that also the female, mated in the first crossing, was most probably homozygous.

Fitness tests

Flies used in these tests were maintained under the same nutritional treatment and had roughly same size within the sexes. The experimental replicates covered at least two generations.

Mating ability. Tests on the transgenic males´ mating ability in comparison to WT under controlled laboratory conditions were run following the manual for Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies (http://www-naweb.iaea.org/nafa/ipc/public/d4_pbl_5_1.html) with some modifications, in plexiglass cages of the size of 30 x 40 x 30 cm (w x d x h), covered on one side with a fine nylon net. Freshly emerged flies from lines #1260_F-3_m-1, #1260_F-1_m-2, #1261_F-5_m-5, #1261_F-5_m-4, and WT were separated by sex to keep them as virgins and provided with food (yeast hydrolysate: sucrose; 1:3) and water. After 48 h, for each transgenic line ten cages were set, each containing 25 WT females, 25 WT males and 25 transgenic males. Ten control cages were also set, each containing 25 WT females and 50 WT males. The flies were allowed to mate for six hours, single mating pairs were removed and transferred into 28 ml cylindrical plastic vials. At the end of the mating, the males were screened by epifluorescence to discriminate WT from transgenics. The data were analyzed according to *G*-tests[12] to verify the homogeneity among the replications within each line and to compare the total mating frequency across the lines. The ratio between females mated to WT or transgenic males was analyzed for statistical significance from the theoretical hypothesis 1:1 by using *G*-tests. Copulation latency was recorded and compared within and between the lines by using *t*-tests.

Adult longevity. Male and female adult longevity was determined daily for the lines #1260_F-3_m-1, #1260_F-1_m-2, #1261_F-5_m-5, #1261_F-5_m-4, and WT. Immediately after eclosion, 25 virgin males and 25 virgin females for each line were singly transferred to a cage and provided with food and water. Counts of dead flies were made every 24 h until all died. For each transgenic line mean daily longevity of both sexes was determined and compared to WT longevity by using *t*-tests.

Paternity success. To test whether transgenic sperm is effectively transferred to and used in WT females, a mating test with transgenic and WT males competing for WT females was performed. Freshly emerged flies from lines #1260_F-3_m-1, #1260_F-1_m-2, #1261_F-5_m-5, #1261_F-5_m-4, and WT were separated and provided with food and water. 48 hours after eclosion, one cage containing 25 WT females, 25 WT males and 25 transgenic males was set for each transgenic line. The flies were maintained for about 15 days in the cages, allowing them to mate and lay eggs. Six batches of eggs oviposed within 24 h were collected every two days. The eclosed progeny were screened by epifluorescence optics for the expression of the *PUb-DsRed*, *PUb-EGFP*, *Ccβ2t-tGFP* and *Ccβ2t-DsRedEx*.

Sperm count. In a separate non-competing experiment the quantity of transferred sperm from transgenic or WT males to WT females was measured. To determine the quantity of transferred sperm in the WT spermathecae, a total of 279 WT females were dissected (50 mated to WT males; 72 mated to #1260_F-3_m-1 males; 61 mated to #1260_F-1_m-2 males; 54 mated to #1261_F-5_m-5 males; 42

mated to #1261_F-5_m-4 males). To avoid any possible incomplete sperm transfer, only spermathecae from females mated for more than 100 minutes were dissected 24 hours after the end of the mating to allow the complete filling of both spermathecae[13]. After dissection, intact spermathecae and their ducts were placed in a 7 µl drop of 1% phosphate buffered saline (PBS) on the well (8 mm Ø) of a microscope multitest slide. Spermathecae were gently broken with fine dissecting pins and the drop was stirred for 1 min to homogeneously spread the sperm. A 5 µl drop of 4 µg/ml 4',6-diamidino-2-phenylindole (DAPI) solution was added and covered with a 12 mm Ø coverslip. After staining for 7 min, the coverslip was secured with a drop of clear nail polish. DAPI-stained sperm heads were counted from the whole slide area using an epifluorescence Zeiss Axioplan microscope at 200x magnification. The data were analyzed by using ANOVA. The raw data were normalized by square root function to reduce the pronounced positive skewness. The number of females whose spermathecae were found empty was also compared among the lines by using *G*-tests.

Hatching rate. Two-day old virgin WT females and WT, #1260_F-3_m-1, #1260_F-1_m-2, #1261_F-5_m-5, or #1261_F-5_m-4 males (in each crossing 75 females: 150 males), were separately placed in mating cages and allowed to mate for six hours. The couples were separately collected and, after a single mating was completed, individual females were transferred to a cage, provided with food and water and allowed to ovipose. The number of WT females mated to males of the different lines were: 33 to WT, 34 to #1260_F-3_m-1, 35 to #1260_F-1_m-2, 37 to #1261_F-5_m-5 and 42 to #1261_F-5_m-4. The eggs laid within 24 h were collected in a 11 cm Ø Petri dish in three separate collections at three, ten and 15 days after the mating. The embryos were maintained in water for 5 days, allowing the larvae to hatch. The number of larvae and unhatched embryos were checked under a binocular microscope. All the data were analyzed by using *G*-tests across the lines.

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3.7 Site-specific integration to modify successfully tested transgenic *Ceratitis capitata* (Diptera: Tephritidae) lines

With this study an unidirectional site-specific integration system should be established in *C. capitata.* Such site-specific integration allows the modification of successfully tested and positively evaluated transgenic medfly lines like described in 3.4 and 3.6. Such modifications could be the addition of marker genes. Furthermore, rearrangements of transgene constructs to make them genetically more stable or to actually change the transgenes completely, can be carried out at defined and already positively evaluated genomic positions.
Molecular verification and the proof of stabilized transgenes will complete the analysis.

Marc F. Schetelig, Francesca Scolari, Alfred M. Handler, Giuliano Gasperi and Ernst A. Wimmer

Author contributions to the practical work:

Marc F. Schetelig: All experiments, and partial* / besides**
Francesca Scolari: *Generation of plasmids #1252 - #1255
Alfred M. Handler: **Generation of jumpstarter lines

STATUS: MANUSCRIPT IN PREPARATION.

Site-specific integration to modify successfully tested transgenic *Ceratitis capitata* (Diptera: Tephritidae) lines

Marc F. Schetelig[1], Francesca Scolari[2], Alfred M. Handler[3], Giuliano Gasperi[2] & Ernst A. Wimmer[1]

[1] Department of Developmental Biology, Georg-August-University Göttingen, GZMB, Johann-Friedrich Blumenbach Institute of Zoology and Anthropology, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany.

[2] Dipartimento di Biologia Animale, Università di Pavia, Piazza Botta 9, 27100 Pavia, Italy.

[3] USDA/ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL, USA.

Author contributions: M.F.S., F.S., A.M.H. designed and constructed the DNA constructs and assisted with the molecular analysis of transgenics. M.F.S. and A.M.H. created the transgenic medflies and M.F.S. conducted the phenotypic and molecular analysis. E.A.W. and G.G. conceived and supervised the project. M.F.S. wrote the paper with E.A.W. All authors discussed the results and commented on the manuscript. Correspondence and requests for materials should be addressed to E.A.W. (e-mail: ewimmer@gwdg.de).

Abstract

Insect transgenesis is mainly based on random integration of DNA constructs embedded into non-autonomous transposable elements (Coates et al., 1998; Handler and Harrell, 2001b; Handler et al., 1998; Jasinskiene et al., 1998; Loukeris et al., 1995). However, once a random integration site has been identified as an advantageous location in the genome, one would like to use that location for further integration of transgenes. Site-specific integration could serve this purpose and has recently been functional in the vinegar fly *Drosophila melanogaster* (*D. melanogaster*) (Bischof et al., 2007; Groth et al., 2004; Venken et al., 2006) and the yellow fever mosquito *Aedes aegypti* (*A. aegypti*) (Nimmo et al., 2006) by applying the integration system of the bacteriophage *phiC31*. Here we show that this site-specific integration system is also functional in the economically important insect pest *Ceratitis capitata* (*C. capitata*; Diptera: Tephritidae). Our results demonstrate the site-specific integration of a construct containing a short 52 bp *attB*-site into a molecularly characterized transgenic medfly line carrying a 51 bp *attP*-site. The site-specific integration shown here allows us to further modify successfully tested and positively evaluated transgenic medfly lines. Such modifications could be the addition of further marker genes. Furthermore, rearrangements of transgene constructs can be carried out at defined and already positively evaluated genomic positions. This can be used to make transgenes genetically more stable or to actually change the transgenes completely.

Keywords: phiC31, *attP*, *attB*, integrase, recombination

Introduction

The ability to genetically transform insects by the use of recombinant DNA allows for a broad range of genetic modifications. For a number of pest species transgenesis is currently used to create strains with different advantages to improve existing biocontrol methods such as the sterile insect technique (SIT) (Condon et al., 2007; Nimmo et al., 2006; Nolan et al., 2002; Schetelig et al., in preparation). During the process of transgenesis constructs based on transposable elements integrate randomly into the genome. However, the integration site often influences the functionality of transgenic constructs due to position effects and is often crucial for the overall fitness of the transgenic lines (Marrelli et al., 2006). Once a healthy and fit transgenic as well as functional line is identified, it would be desirable to further use or modify its genomic situation in order to combine it with other transgenic systems. To implement such modifications at characterized genomic positions, a site-specific integration system is necessary and some of its components have to be integrated already during the initial transposition event. A possible unidirectional system, which is ideal for obtaining efficient and stable integrations, is the phiC31-mediated site-specific integration system (Thorpe et al., 2000). In *D. melanogaster* this system was successfully used to generate transgenic lines (Groth et al., 2004) and to stably integrate DNA fragments larger than 100 kb at specific *attachment P* (*attP*) sites into the genome (Venken et al., 2006). Moreover, the phiC31-mediated integration system was established for a relevent pest species, the yellow fever mosquito *A.aegypti* (Nimmo et al., 2006). Here we report the first use of the phiC31-mediated site-specific integration system in the insect pest *C. capitata*. Due to the design of the integrating constructs an additional transposable end can be added to the two existing ones from the initial transposition (Fig. 1), allowing the deletion of transposable ends by subsequent transposition events. This can further increase the stability of transgenic lines generated by germline transformation using transposable elements. Such genetically stable lines are important for applied applications such as SIT programs, in which lines have to fulfill additional qualifications regarding transgene stability compared to laboratory lines used only for research in laboratories.

Results

To assess site-specific integration in medfly, the characterized transgenic line *TREhs43-hid*^{Ala5}_F1m1 (Schetelig et al., in preparation) containing a 51 bp *attP* recombination site was used for integration experiments. This line carries a polyubiquitin promoter (PUb) driving EGFP expression in the adult thorax and was generated by *piggyBac*-mediated transformation. The green fluorescent thorax pattern is not due to a thorax-specific function of the PUb, but rather results from the integration site of this construct in the genome. In previous studies it was already shown that this PUb promoter could produce various green fluorescent patterns depending on neighboring enhancer elements due to

different genomic integrations sites (Handler and Harrell, 2001a). We coinjected the integration construct pSL*af_3´pBac-attB_PUb-DsRed_af* (#1252) together with capped integrase mRNA from *pcDNA3.1-phiC31* (Bischof et al., 2007) into medfly embryos of the *attP*-containing line *TREhs43-hid*^{Ala5}_F1m1. In G_0 adults we detected randomly distributed strong red fluorescent spots in four of 25 adult flies, indicating a mosaic integration of the *PUb-DsRed* already in G_0. All 25 adults were separated by sex and mated in six groups of two to six G_0-individuals to ten WT flies of the opposite sex.

In case of site-specific integration the position effects, enhancing the PUb promoter-mediated expression of EGFP are supposed to control also the second PUb promoter-driven expression of DsRed. Thus, the red and green fluorescent expression pattern should be similar. Progeny from the mating no. 6 (two G_0 females mated to ten WT males) showed a red fluorescent thorax (Fig. 1b), similar in shape and intensity to the original green fluorescent thorax pattern (Fig. 1a). The resulting line (int6) was successively outcrossed twice to WT flies. Half the progeny of these crossings were double fluorescent (red and green thorax), while the other half were non-fluorescent, showing a genetic linkage of the two fluorescent markers. This was the case for transgenic males crossed to WT females and transgenic females crossed to WT males. To generate a homozygous int6 line, we inbred heterozygous individuals. From 700 adults, we identified 178 (25,4 %) as homozygous for green and red fluorescence by intensity and inbred these as homozygous int6 line. The other adults were identified as heterozygous for red and green fluorescence (347 adults = 49,6 %) or non-fluorescent (175 adults = 25, 0%). No adults showed a segregation of red and green fluorescence, indicating the close proximity of their genomic localization.

The integrated construct #1252 carried an additional 3´ *piggyBac*-end (Fig. 1a), creating a genomic situation with three *piggyBac*-ends (Fig. 1b). This could be used to subsequently delete the 5´ and one 3´ *piggyBac*-end by crossing the int6 line to a functional *piggyBac* jumpstarter line. To test this possibility, we generated several jumpstarter lines. Three of them (jump_1, jump_3, and jump_4) successfully remobilized *piggyBac*-mediated insertions (of the construct p*Bac{sl1-tTA_PUb-DsRed}*; (Schetelig et al., in preparation)) as indicated by novel expression patterns of the PUb-DsRed marker (supplementary Figure 1). The homozygous jumpstarter line jump_3 was crossed to the homozygous int6 line in three independent crossings (1f, 2m, and 3f). Progeny was separated by sex and outcrossed to identify jumpstarter-free individuals. Deletion or other transposition events caused by the transposase source were detected in G_1 progeny by loss or change of fluorescent patterns. From the three crossings 1f, 2m, and 3f, we identified 3-6 % of flies showing the green, but not red fluorescent thorax. This showed the successful excision of the red marker (Fig. 1c). From each independent crossing, one line with green fluorescent thorax only was inbred and brought to homozygous condition (1f-save, 2m-save, and 3f-save). In addition to the deletion of part of the integrated construct, we identified in each crossing several combinations of transposition and reintegration events due to the three *piggyBac*-ends (Fig. 2). In general, a 5´ and 3´ *piggyBac* end and a *piggyBac* transposase source

are needed for a successful transposition event. In medfly, endogenous *piggyBac* elements have not been reported so far. The ability to independently excise or mobilize the red fluorescent marker by *piggyBac* transposition indicates the close proximity of the newly introduced DsRed marker together with the 3´ *piggyBac* end to the previously integrated 5´ *piggyBac* end, which therefore confirms the site-specific integration. At the same time this deletion mechanism leaves a transgene with only one transposable element in the genome. This will reduce the probability of accidental transposition events, which increases the stability of such transgenes and thus the safety against unintentional horizontal transfer.

Discussion

In conclusion, this study describes the first phiC31-mediated site-specific integration in medfly. It will simplify the combination of different transgenic systems in this pest species and might even permit the integration of large DNA fragments as has been reported for *D. melanogaster* (Venken et al., 2006). An advantage of generating transgene combinations via the phiC31-mediated system over other homologous integration systems like Cre/lox or FRT/Flp is the unidirectional integration (Branda and Dymecki, 2004; Wimmer, 2005). In contrast to other systems, integration and excision are mediated by two different enzymes – the integrase and the excisase, respectively. Thus, the phiC31-mediated integration is not reversible by the integrase itself and generates stable transgenes immediately after integration. For pest management programs it is e.g. conceivable to combine characterized conditional embryonic lethal lines (Schetelig et al., in preparation) with a sperm marking system (Scolari et al., 2008) and to subsequently increase the stability of the line by deleting *piggyBac* ends. phiC31-mediated site-specific integration allows to generate lines with transgenes in contiguity and an inheritance of the combined transgenes into next generations as one construct. This will speed up the generation of homozygous lines and simplify the quality control for lines both in the laboratory and in applied medfly programs. In addition, integration sites that have been positively evaluated can be used to further modify transgenes. The site-specific integration system will help to improve stability of such characterized transgenic systems by deleting transposon ends and will reduce the efforts traditionally needed to identify suitable lines generated by random integration. Thus, it will be easier to obtain healthy and fit lines without interfering position effects.

Methods

Plasmid construction

The *attB*-containing constructs pSL*af_3´pBac-attB_PUb-DsRed_af* (#1252) and pSL*af_3´pBac->-attB_PUb-DsRed_af* (#1253) or pSL*af_3´pBac-attB_PUb-EGFP_af* (#1254) and pSL*af_3´pBac->-attB_PUb-EGFP_af* (#1255) were generated by ligating the *Bgl*II/*Afl*II cut fragment *PUb-DsRed* (3.0 kb) or *PUb-EGFP* (3.2 kb) from #1200 (Scolari et al., 2008) or #1201 (Scolari et al., 2008), respectively in the *Bgl*II/*Afl*II cut vectors pSLaf*_3´pBac-attB*_af (#1250) and pSLaf*_3´pBac->-attB*_af (#1251). We created #1250 or #1251 by cloning hybridized primers mfs-205 (GATCCTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACCTCACA)/ mfs-206 (GATCTGTGAGGTGGAGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCAG) in the *Bam*HI/*Bgl*II site of pSLaf*_3´pBac*_af or *pSLaf_3´pBac->_af*, respectively. The *pSLaf_3´pBac->_af* was generated by ligating a 0.4 kb gypsy element, amplified by PCR on p*Bac{3xP3-DsRed>af}* (Sarkar et al., 2006) with the primer pair mfs-197 (CAGTGGGCCCGGTACCCTATTCGCAAAAAC)/ mfs-198 (ACTCGGATCCGGCTAAATGGTATGGCAAG) and subsequent digestion with restriction enzymes *Apa*I/*Bam*HI, in the *Apa*I/*Bam*HI cut pSLaf*_3´pBac*_af. To generate pSLaf*_3´pBac*_af, an 1.3 kb *3´pBac Eco*RV/ *Hpa*I fragment from p3E1.2 was ligated into the *Sma*I cut pSLfa1180fa (Horn and Wimmer, 2000).

Each of the *pBac*-jumpstarter lines contains the pMi{Ccwhite+; hspBac} (AMH_370) generated by an exchange of the 3xP3-DsRed from pMi{3xP3-DsRed; hsp70-piggyBac} (Horn et al., 2003) for the medfly *white* gene.

Medfly transformation and rearing

The medfly lines *we,wp* (phenotypic: white eyes, white pupae) and WT (EgII) were received from Gerald Franz (IAEA, Seibersdorf, Austria). Medfly transformation using *Minos* transposable elements marked by *hsCcw* (Loukeris et al., 1995) to generate *piggyBac* jumpstarter lines was performed using standard methods (Handler and James, 2000) into the *we,wp* strain. The capped phiC31 integrase mRNA was transcribed from the *Bam*HI cut plasmid *pcDNA3.1-phiC31* (Bischof et al., 2007) according to the protocol of the mMESSAGE mMACHINE T7 kit (Ambion, Austin). Coinjection of the *attB*-containing plasmid #1252 and capped integrase mRNA into the *attP*-containing line *TREhs43-hid*^{Ala5}_F1m1 was done as described (Groth et al., 2004). The line *TREhs43-hid*^{Ala5}_F1m1 was previously described (Schetelig et al., in preparation).

Remobilization assay

Three medfly lines were used to perform the remobilization assay: int6, jump_3, and *we,wp*. int6 carries both the genetically linked red and green fluorescent markers in homozygous condition. jump_3 carries in *we* background the genetically linked medfly *hsCcw* marker gene (Loukeris et al.,

1995) and the *piggyBac* transposase in homozygous condition. To remobilize transgenes together with their transposable ends the homozygous int6 line (Fig. 1b) was crossed to the homozygous jumpstarter line jump_3. Progeny, heterozygous for both markers and the *piggyBac* transposase, was outcrossed to *we,wp*. Their progeny was screened for green and red fluorescent expression patterns. White eyes indicated the absence of the genetically linked *hsCcw* marker and the *piggyBac* transposase genes. Successful remobilization and reintegration events were identified by new expression patterns of the PUb markers (Fig. 1 and 2). A loss of the red marker only indicated a successful excision without new integration (Fig. 1c).

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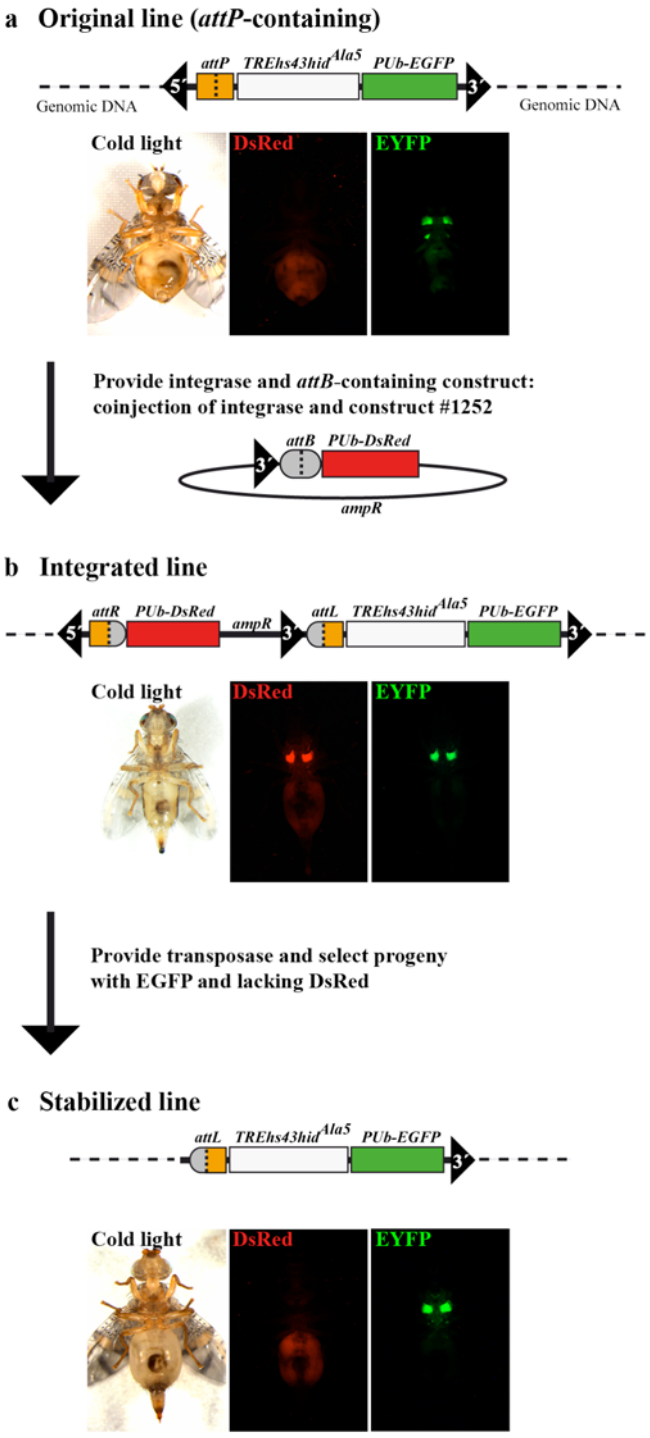

Figure 1. Scheme for achieving transgene stability using a combination of site-specific integration and subsequent transposition events

Diagrams show relative positions of 5´ and 3´ *piggyBac* terminal inverted repeats, the PUb-EGFP and PUb-DsRed markers, and the *attachment-P*, *-B*, *-L*, and *-R* sites. Images show the original line *TREhs43-hid*^{Ala5}_F1m1 (a), the integrated line int6 (b) and the stabilized line 2m-save (c). Flies were observed with different filter sets: cold light, DsRed filter (Ext. 565/30; Emm. 620/60), and EYFP filter (Ext. 500/20; Emm. 535/30).

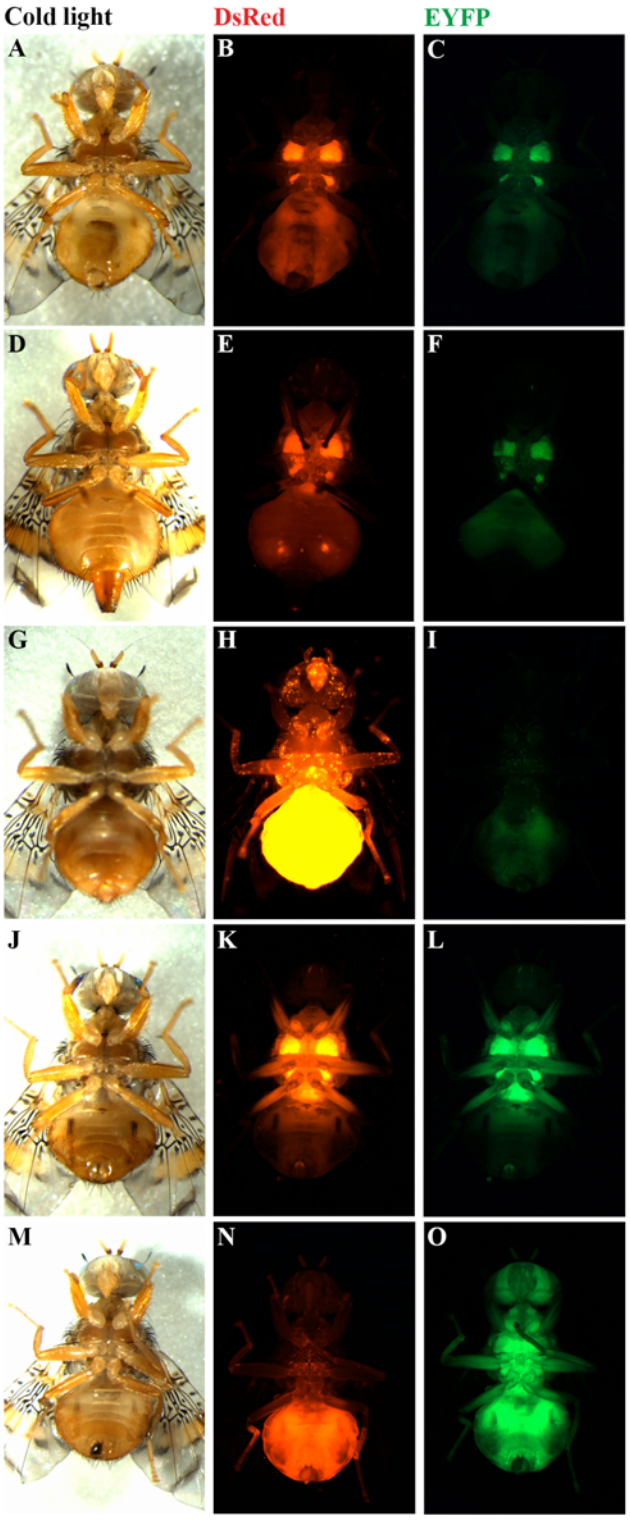

Figure 2. Remobilization events from matings of the integrated line int6 to the jumpstarter line jump_3

Each fly was observed with different filter sets: cold light, DsRedwide filter (Ext. 546/12; Emm. 605/75), and EYFP filter (Ext. 500/20; Emm. 535/30). (A-C) Thoracic red and green fluorescence similar to the expression pattern of the original int6 line. (D-F) Original integration with red and green thorax kept plus new integration of the red marker only in the abdomen. (G-I) Loss of original integration and new integration of only the red marker in the head, thorax, legs, and abdomen. (J-L) New integration of both markers together (red and green legs as well as thorax). (M-O) New independent integrations of the red (abdomen) and the green marker (head, thorax, legs and abdomen), potentially by successive remobilization and excision events.

Supplementary

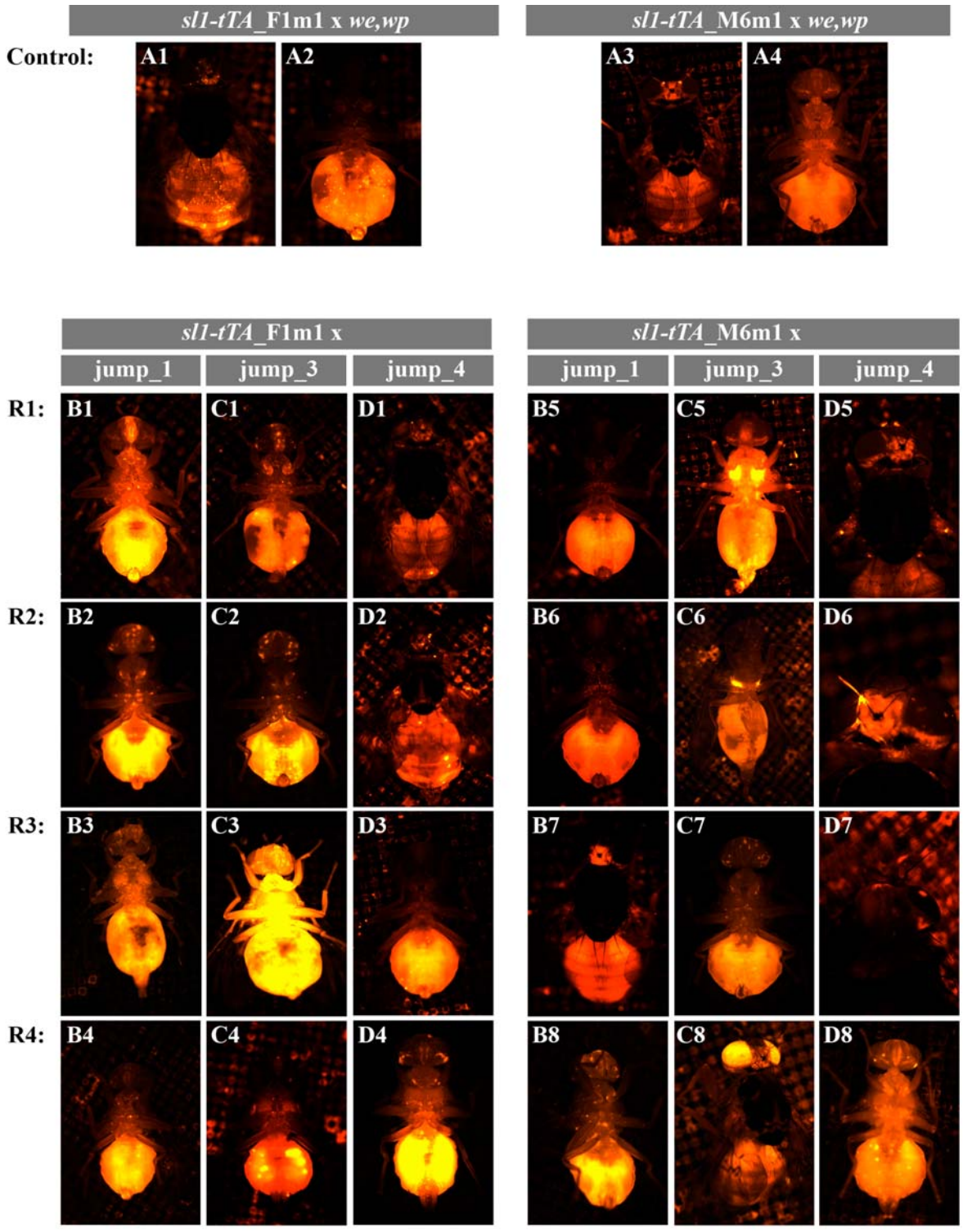

Figure S1. Jumpstarter test

To generate lines homozygous for a red fluorescent marker and white eyes, the lines *sl1-tTA*_F1m1 and *sl1-tTA*_M6m1 (carrying a PUb-DsRed marker) were crossed to the line *we,wp* (white eye, white pupae). Heterozygous progeny of both crossings was inbred and their progeny screened for the homozygous red fluorescent marker and white eyes.

To test the efficiency of medfly *piggyBac*-jumpstarter lines, a male from the generated homozygous medfly lines *sl1-tTA*_F1_*PUbDsRed,we,wp* or *sl1-tTA*_M6_*PUbDsRed,we,wp* was mated in four repetitions (**R1-R4**) to four females of the three independent homozygous jumpstarter-lines jump_1 (**B1-B8**), jump_3 (**C1-C8**) or jump_4 (**D1-D8**), respectively. G1 males, heterozygous for the red marker and the *piggyBac* transposase, were outcrossed to *we,wp* females and progeny was analyzed for new red fluorescent patterns indicating successful remobilization events. White eyes indicated the loss of transposase source. From each crossing, an adult with fluorescent patterns different from the patterns of the heterozygous control adults (**A1-A4**) represent successful remobilization due to the transposase source. Control adults: red fluorescent expression pattern of heterozygous adults from the lines *sl1-tTA*_F1_*PUbDsRed,we,wp* and *sl1-tTA*_M6_*PUbDsRed,we,wp* crossed to *we,wp* (dorsal view: A1 and A3; ventral view: A2 and A4). Flies were observed with DsRedwide filter (Ext. 546/12; Emm. 605/75).

4 Discussion

In this study, transgenic systems to improve several aspects of medfly SIT programs have been developed and evaluated. Especially the conditional embryonic lethality (see 3.4) and the sperm marking systems (see 3.6) or a combination thereof, which should be possible with the *phiC31*-mediated site-specific integration system (see 3.7), will improve the sterilization, marking, and monitoring during SIT programs. However, the use of transgenic individuals for field applications like SIT programs would be a novum and thus the generated systems need to be discussed in this repect.

4.1 Sperm marking and sexing systems

To monitor the efficiency of an SIT program and manage subsequent releases, marking systems for released pest species are needed. The developed transgenic sperm marking (see 3.6) could serve as a major improvement for the monitoring within medfly SIT programs. The use of such harmless transgenic markers will serve as an ideal initial condition to transfer insect transgenesis technology from the laboratory to field applications. If the sperm-marked medfly strains are used, the marking step by dusts at the end of the mass-rearing process could be skipped and labor input reduced. Moreover, effective and easily recognizable sperm-marking will make novel studies possible on medfly reproductive biology, which will help to further improve SIT programs. In principle, as shown for *Anopheles stephensi* (Catteruccia et al., 2005), a sperm-marker can even be used for sexing by automatic larval sorting. But the testes-specific fluorescence of the generated medfly strains was not detected in early larval stages and not in about 50% of last stage larvae. Thus, sexing of larval stages with the developed system does not make sense so far. To generate a transgenic sexing system for medfly by using the sperm-marking system, improvements have to be achieved first. Multiple insertions or a different insertion site of the sperm marker could help to generate a strain with strong larval fluorescence. Nevertheless, multiple insertions and other insertion sites could also cause fitness problems or difficulties during the quality control to maintain homozygous strains.

Other methods for sexing flies have been shown in *D. melanogaster*. A tetracycline-repressible female-specific lethal genetic system was successfully tested in *D. melanogaster* (Heinrich and Scott, 2000), but could not be transferred to *C. capitata* so far. Another method transformed female *C. capitata* into males (Pane et al., 2002). In principle such a method is able to double the amount of produced males and therefore improve production capacities enormously. Transferring the system from the transient to a transgenic RNA interference mediating situation generated “only” 95% males and 5% intersexes (Saccone et al., 2007). However, SIT programs require competitive males. Thus, tests have to show the effects of transgenic intersex flies, XY males, and especially the XX males compared to normal XY males.

Recently transgenic sexing was demonstrated in medfly using a lethality system combined with female-specific alternative splicing (Fu et al., 2007). This transgenic sexing system kills female progeny effectively but not until the late larval stage. Larvae and adults would therefore develop unintentionally during mass rearing. With the developed embryonic lethality system for medfly (see 3.4) it should be possible to solve this problem of late lethality. Therefore, the embryonic lethality system should be combined with the ability of female-specific splicing (see 3.5), which would generate a female-specific lethality already during embryogenesis. However, first results of this combined system showed only little female-specific lethality. This might be due to position effects of the integrated constructs, which often have dramatic effects on the functionality of transgenic systems. As an example: embryonic lethality systems descending from the same effector line, but with their driver construct at different genomic positions, vary in the degree of lethality from not detectable to 100% (see 3.4). Therefore it might be possible to develop female-embryo-specific lethality by generating lines with the female-specific spliced driver construct at other genomic positions. This could be achieved by new integrations via injection of the driver construct and a transposase source or by crossing the generated female-specific spliced line *sryα-Cctra-tTA*_F1 to an evaluated jumpstarter line (see 3.5). A third option could be a site-specific integration (see 3.7) of a female-specific spliced driver construct directly into the functional effector line *TREhs43_hid*^{Ala5}_F1m2 of the embryonic lethality system (see 3.4). The effector line already carries the necessary *attP* site for site-specific integration and the genomic position was shown to be a suitable position at least for the effector construct of the embryonic lethality system. Therefore the integration of the driver construct at this characterized position might result in an effective, female-specific lethality system. Additionally the exact position of the female specific-spliced *CctraI* seemed to be important for the efficiency and specificity of the splicing (Fu et al., 2007). Thus, new positions of the *CctraI* in the driver or the effector construct of the embryonic lethality system might help to generate complete female-specific lethality.

4.2 Reproductive sterility for *Ceratitis capitata*

Today, the method of choice for producing reproductively sterile insects for releases within AW-IPM programs is the sterilization by radiation. Important for this process is a tight control of the absorbed radiation dose, which ensures sufficiently sterile and yet competitive insects (Bakri et al., 2005). Also other factors like insect age or oxygen levels influence the required absorbed dose and the competitiveness of the insects. In contrast, the established embryonic lethality system for medfly (see 3.4) inherits suppressible reproductive sterility. Therefore the complete radiation process with all its safety and quality assurance could be skipped. By using the embryonic lethal strains instead of irradiated strains, possible risks coming from isotopic sources could be eliminated for workers and the environment.

A second important aspect for SIT programs is the embryonic lethality. A first transgenic system for medfly based on an autoexpression-loop for the tTA protein caused lethality starting only in late larval stages (Gong et al., 2005). Thus, larvae would hatch, feed on the infested fruits, and reduce the fruit quality. In contrast, the newly developed embryonically lethal medfly strains produce reproductively sterile males, which mate to wild type females after release and their progeny die already during embryogenesis ("embryonic lethal"). This inhibits larval hatching. Matings of released reproductively sterile males to wild type females therefore would prevent fruit damage (see 3.4).

Such a transgenic embryonic lethality system is also ideal for preventional control programs, where sterilized insects are released into pest free areas and have to be 100% sterile. The generated embryonic lethality strains showed this 100% sterility in laboratory and field cage tests (see 3.4). In addition, medfly males with the embryonic lethality system were highly competitive in laboratory and field cage tests. The tested strains carry two fluorescent body markers, which could be used immediately for monitoring released males or females. In the future, a combination of the embryonic lethality system with the developed sperm marker would further improve this monitoring aspect (4.1). In contrast, generating sterility by radiation comes along with reduced competitiveness in several species and in medfly the dose of irradiation is fundamental to the degree of sterility but indirectly correlates with competitiveness (Parker and Mehta, 2007). Therefore the medfly embryonic lethality system should become a major improvement for SIT programs, if mass-rearing tests confirm the applicability of the embryonically lethal lines.

The embryonic lethality system might be directly transferable to other Tephritids. Therefore it will be critical, whether the promoter of the cellularization specific gene *C. capitata serendipity α* is functionally conserved. It has been shown that e.g. the *D. melanogaster serendipity α* promoter was not active in medfly, probably because of low conservation of the cellularization genes and its promoters between Drosophilids and Tephritids (see 3.3). The conservation of these genes might be higher within the Tephritids and therefore a direct transfer might be possible. The second possibility to transfer the system to other Tephritids is the use of respective endogenous promoters. However, most of the key pest Tephritid genomes are not sequenced and an easy identification of endogenous non-conserved genes and their promoters might be complicated. But the newly isolated early embryonic medfly genes (see 3.2 and 3.4) might help to isolate their homologs from other Tephritids.

Nevertheless, the low functional conservation of promoters from cellularization genes used in the embryonic lethal strains could also imply a lowered risk in the case of an accidental gene transfer. Such a gene transfer is anyway unlikely, because the embryonically lethal strains are reproductively sterile. In addition, the embryonic lethality system does not carry positively selectable components (e.g. resistances) and therefore a gene transfer should not be of any advantage. But generating transgenic individuals for applied science has to be discussed in respect to all instances to design a system, which is the safest regarding the state of the technology. E.g. the phospho-acceptor-site mutant allele of the proapoptotic gene hid^{Ala5} from *D. melanogaster* (Bergmann et al., 1998) could be

exchanged for endogenous proapoptotic or apoptotic genes to further increase the species specificity. Another possibility for causing lethality would be transgenic RNAi. In that direction, first transient embryonic RNAi experiments with parts of the cellularization genes *C.c. slow as molasses* and *C.c. serendipity α* have shown complete lethality in *C. capitata* embryos (data not shown). Therefore, an approach using transgenic RNAi to cause embryonic lethality in medfly is feasible and would further increase the species specificity.

4.3 Site-specific integration and combination of transgenic systems

In *D. melanogaster* recent technologies enable easier gene manipulation than in any other multicellular organism (Venken and Bellen, 2005). For the insect pest medfly new methods for gene manipulation would help to create molecularly designed deletions, new transgenic approaches and the possibility of cloning and modifying large DNA fragments. With the establishment of the phiC31-mediated site-specific integration in medfly (see 3.7) doors are open to further improve or modify positively evaluated transgenic medfly lines.

One possibility is the combination of different transgenic systems. With the site-specific integration system it is possible to carry out the combination at characterized positions in the genome of successfully evaluated transgenic lines, which afterwards might eliminate the need for labor-intensive evaluations of transgenic lines to find once again a healthy and fit one. For medfly SIT programs it will be particularly interesting to combine the newly generated embryonic lethality (see 3.4), the sperm marking (see 3.6), and a sexing system to improve the sterilization, monitoring, and sexing procedure during SIT programs. For the embryonic lethality and the sperm marking system, positively evaluated transgenic lines have been identified and carry the required *attP* (Groth et al., 2004) site for site-specific integration. Additionally one embryonic lethal line (#67; see 3.4) was successfully tested in field cage tests for competitiveness and might therefore represent the initial line for integrating other transgenic systems. However, the only functional transgenic sexing strain (TSS) for medfly (Fu et al., 2007), which can be used for a combination at present, is not ideal for SIT applications. The females of this system are killed starting in late larval stage, which would increase the costs during the mass rearing compared to the common genetic sexing strains (GSSs). For replacing GSSs, a transgenic sexing system for medfly should be able to kill the females already during embryogenesis. Such a transgenic system does not exist right now, but the isolation of early embryonic active genes and their promoters (see 3.2 and 3.4) will help in the development of such a transgenic embryonic sexing system (see 3.5).

Currently, combination of the transgenic systems of embryonic lethality (see 3.4) and sperm marking (see 3.6) with a well-established GSS like *Vienna-7* or *Vienna-8* (Franz, 2005) is desirable. In this scenario, the sperm marker could be site-specifically integrated into the positively evaluated embryonic lethal line #67. Because the transgenes of the embryonic lethality system are located on

chromosome 5, this integration would generate a situation with all transgenes located on chromosome 5. The currently used GSSs carry a *temperature sensitive lethal* (*tsl*) mutation in a way that female embryos can be killed by heat shock. The wild type allele of the *tsl* is physically linked to the Y-chromosome by a Y-autosome translocation to link the inheritance of this mutation to sex. The mutation of the *tsl* is located on chromosome 5 and it had been shown that the structure of these translocations is crucial for the stability and productivity of the GSSs (Franz, 2005). Thus, a system combining embryonic lethality, sperm marking and a genetic sexing system on chromosome 5 could be generated by site-specific integration and classical genetics. The location of all systems on chromosome 5 could help to minimize the effort of quality control for keeping mass rearing lines in homozygous conditions. In addition, the fluorescent markers of each transgenic system along with the *wp* marker of the GSS are visible markers, which can be used for improving the quality control and the identification of recombinants in mass rearing populations.

Generating multi-functional transgenic lines should also include a strategy of increasing the stability of transgenes. One way of stabilizing a transgene is the combination of the site-specific integration and subsequent transposition events, which eliminates transposable ends. Lines generated that way carry only one transposable end. This genetic situation would no longer be point of action for a transposase source like *piggyBac*, which requires always two transposable ends for successful transposition, and therefore the stability of such transgenic lines should be increased. In flies site-specific genomic targeting to enhance ecological safety of transgenic lines has recently been shown for *D. melanogaster* (Horn and Handler, 2005) and the pest species medfly (Dafa'alla et al., 2006). Both systems are based on homologous integration systems with size limitations for the integrated DNA fragments. With the phiC31-mediated integration system for medfly it might be possible to integrate larger DNA fragments like demonstrated for *D. melanogaster* (Venken et al., 2006) and the unidirectional mechanism would allow for the generation of stable transgenes immediately after integration.

Thus, all advantages of existing and future transgenic systems could be combined in medfly by the site-specific integration system at defined and characterized positions and in addition the stability of transgenes might be increased.

4.4 Biotechnology in applied sciences

4.4.1 Resistance to the SIT and/or transgenic engineered lines?

Using the SIT for AW-IPM programs raises the questions: “Can insects develop resistance to the SIT?” - and in the case of using transgenic-engineered systems for SIT - “Does the use of transgenic individuals for SIT pose an increased risk?” A considered kind of resistance to the SIT is that a target species could evolve mechanisms to avoid mass-reared and sterilized males. This might happen for every mass-reared strain regardless of transgenic or non-transgenic. To ensure the competitiveness of mass-reared insects to their wild counterparts, a re-colonization of a population from the field and

replacing the original mass-rearing colony could be carried out. This could become more problematic if specialized strains like the medfly GSSs are used (Robinson et al., 1999). For specialized strains the competitiveness of males has to be constantly monitored by field cage tests under semi-natural conditions to ensure the quality of the mass-rearing mother colonies. A method to preserve specialized mass-rearing strains at a time, where they have been successfully tested for competitiveness, might be the cryo-preservation of embryos. This method reversibly arrests embryos in the embryogenesis so that they can be stored for several months up to some years. Preserved strains could then be used for regular replacement of a mass-rearing colony and therefore prevent resistances from being established in the mass-rearing colony. For some species of economic importance this technique was already developed: New World Screwworm (Leopold et al., 2001) and medfly (Rajamohan et al., 2003). Nevertheless, AW-IPM programs integrating SIT for New World screwworm or medfly are active for decades and so far there is no evidence that "resistance to SIT" has developed (Krafsur, 1998).

The key difference of molecular sterility to radiation based sterility, in which DNA breaks are randomly induced all over the chromosomes by radiation, is that molecular sterility is based on one or few dominant factors. Any variations in the expression of the dominant factor (e.g. the lethality inducing factor hid^{Ala5}) might lead to a natural selection of wild type females after the interaction with the released males giving rise to non- or less-sensitive progeny. This could then lead to a loss of sterility induction in subsequent releases. One way to overcome such a situation could be the introduction of multiple diverse and unrelated lethal effectors, but fitness costs of such transgenic lines have to be determined. However, proapoptotic genes are functionally highly conserved and apoptosis is a regular developmental process, which is needed for correct development. Thus, proapoptotic genes like hid^{Ala5} should be no targets for natural selection.

Another scenario of biological resistance can be considered for transgenic systems, which use transgene combinations suppressible by an antibiotic. In the field of insect biology, resistances to pesticides, insecticides, or antibiotics are mainly based on enzymatic deactivation or reduced drug accumulation. Both mechanisms reduce the sensitivity to the compounds. By natural selection the resistances then spread in the population. So this process would rather appear during the mass rearing than during release, because released insects are reproductively sterile, can not become established into the environment, and are therefore no targets of natural selection as known from some transgenically engineered plants. Mass-reared transgenic lines (see 3.4, (Gong et al., 2005), (Fu et al., 2007)), which are reared on food that contains antibiotics e.g. for the suppression of conditional lethality, might be targets of natural selection. In that case, the target is supposed to be the gut flora of the insects. Therefore a classical antibiotic resistance of bacteria to the food supplement rather than the transgenic system itself has to be considered. As an example, the antibiotic tetracycline is used for the suppression of the embryonic lethality of medfly during the rearing process. In the case that bacteria in the gut evolve in the direction that they would be able to destroy the tetracycline, the suppression of lethality could be weakened. Then a higher dose of tetracycline has to be used, which was shown to be

critical for the overall fitness of flies at higher concentrations (see 3.4) or a functional tetracycline analog could be used for the suppression. Most of these analogs are expensive and therefore no reasonable alternatives for mass production of insects. The better alternative to overcome this situation would be the regeneration of the transgenic lines by crossing driver and effector lines once again to regenerate the embryonic lethality line. Since the driver and the effector lines of the embryonic lethality system (see 3.4) can be reared without tetracycline, they are not target of natural selection towards tetracycline-resistant individuals. The regenerated embryonic lethality lines could then replace a mass-rearing colony, which might have developed the described antibiotic resistance. The possibility to regenerate such lines is therefore an advantage of a two-component system (see 3.4) over one-component systems (Fu et al., 2007; Gong et al., 2005), which have to be reared with tetracycline as a food supplement at any time to suppress the lethality under rearing conditions. Nevertheless, the long-term stability and feasibility to use transgenic systems for SIT has to be shown in large-scale field tests in the future.

4.4.2 Conventional vs. transgenic improvements

Approaches based on selecting natural mutants (e.g. the medfly phenotypical marker *white pupae*) or on classical genetics (e.g. the medfly *temperature sensitive lethal* sexing strain *Vienna-8* (Franz, 2005)) have been proven to be useful tools to improve the SIT. Nevertheless the isolation of natural mutants to use for special purposes is often based on serendipity and is labor intensive. In almost the same manner it is difficult and time-consuming to generate fly strains with distinct special qualities. Improving the SIT by the use of transgenic systems is an alternative to such methods. With the help of transgenic methodology the design of fly strains with specific characteristics is possible in a relatively short time compared to other methods attempting something similar. In addition, transgenic systems should be easier to transfer from one species to another compared to the development of classical genetic systems in different species. Even if a transgenic system was generated highly specific for one species, it will be possible to modify and generate the system for a new target species using the transgenic methodology.

There are ongoing concerns about using biotechnology for field applications. But the initial position of using transgenic technology for SIT is rather different to the use of biotechnology in other areas. The developed systems for sperm marking and embryonic lethality do not carry positively selectable elements such as resistances and thus differ fundamentally e.g. from plant biotechnology, where transgenically produced insecticides (Tabashnik et al., 2006; Tabashnik et al., 2008) or herbicide resistances are used (Gasser and Fraley, 1989). Another fundamental difference e.g. to transgenic plants is, that released sterile insects of SIT programs are reproductively sterile and therefore do not inherit their genetic background into wild populations. In this respect it has to be ensured that the released insects are completely sterile no matter if sterilized by radiation or using autocidal control with transgenic technology (even if fly strains engineered by classic genetics are not declared as

GMOs). The developed embryonic lethality system for medfly shows 100% lethality in laboratory tests as well as in field cage tests (see 3.4). Thus, the use of this system would ensure that no transgenic material is transferred into wild populations.

An accidential escape of *Ceratitis* from mass-rearing facilities would currently cause problems, if the insects have not been sterilized before. However, by using the embryonic lethal lines, the escaped insects would be 100% reproductively sterile. Thus they would not cause any problems even when escaped into preventional areas, where the insect pest is not present. In this direction, transgenic insects can increase the safety of the mass-rearing process for operational SIT programs.

Another concern is the eradication of species during SIT programs. Most of the species declared as pest species are invasive species. Medfly e.g. originally comes from Kenya and was spread worldwide by distributive trades. That's why control programs against such invasive species rather provide for the biological balance than disrupt it. In addition, the term "eradication" in the SIT does not describe the worldwide elimination of a species, but more their local elimination. Thus, the concern about eradication programs does not fit to the medfly control programs. But eradication programs for non-invasive species should be clearly discussed. *Anopheles gambiae* (*A. gambiae*) is one prominent example. On the one hand *A. gambiae* is the carrier of malaria and it is desirable to eliminate this disease. On the other hand *A. gambiae* is a native species in several countries and therefore a total eradication of this mosquito could cause environmental problems. Here, IPM shows again its potentials of combining different methods to find the most biocompatible solution for different areas: for *A. gambiae* the combined use of vaccines, drugs, different control methods, and suppression programs will hopefully be a powerful alternative to total eradication. In general, IPM using a spectrum of different control mechanisms including the powerful transgenic technology could enhance an environmental-friendly pest control and should be tested in the future.

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6 Appendix

6.1 Abbrevations

A. gambiae	*Anopheles gambiae*
A. suspensa	*Anastrepha suspensa* / caribfly / Caribbean fruit fly
AW-IPM	area-wide integrated pest management
C. capitata	*Ceratitis capitata* / medfly / Mediterranean fruit fly
CctraI	female-specific spliced intron from the *C. capitata* gene *transformer*
D. melanogaster	*Drosophila melanogaster*
DsRed	*Discosoma* species red fluorescent protein
EGFP	enhanced green fluorescent protein
GSS	genetic sexing strain
IPM	integrated pest management
RNAi	RNA interference
SIT	sterile insect technique
TRE	tTA-responsive element
tTA	tetracycline-controlled transactivator
TSS	transgenic sexing strain
WT	wild type

6.2 Sequences

All sequences are shown in GenBank format and are mentioned in chapter 3.4.

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            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 418)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
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  TITLE     Conditional embryonic lethality to improve the sterile insect
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 5781)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
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5761 accaaaaaaa aaaaaaaaaa a
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LOCUS       upstream_Cc_sub2-99      661 bp    DNA     linear
DEFINITION  Ceratitis capitata sub2-99 (Cc_sub2-99) gene, 5' UTR and partial
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ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 661)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 661)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
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LOCUS       Cc_sub2-99              1876 bp    mRNA    linear
DEFINITION  Ceratitis capitata sub2-99 (Cc_sub2-99) cDNA, 5'UTR and partial
            CDS.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 1876)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1876)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
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1861 aaaaaaaaaa aaaaaa
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LOCUS       upstream_Cc_CG2186      1516 bp    DNA     linear
DEFINITION  Ceratitis capitata CG2186 (Cc_CG2186) gene, 5' UTR and partial cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 1516)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1516)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
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LOCUS       Cc_CG2186               3329 bp    mRNA    linear
DEFINITION  Ceratitis capitata CG2186 (Cc_CG2186) cDNA, complete cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 3329)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 3329)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
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LOCUS       upstream_Cc_srya        1674 bp    DNA     linear
DEFINITION  Ceratitis capitata serendipity alpha (Cc_srya) gene, 5' UTR and partial
cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 1674)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1674)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
FEATURES             Location/Qualifiers
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LOCUS       Cc_srya                 2526 bp    mRNA    linear
DEFINITION  Ceratitis capitata serendipity alpha (Cc_srya) cDNA, complete cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 2526)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2526)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
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```
LOCUS       upstream_Cc_sub2-63     1406 bp    DNA     linear
DEFINITION  Ceratitis capitata sub2-63 (Cc_sub2-63) gene, 5' UTR and partial cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 1406)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1406)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
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LOCUS       Cc_sub2-63              2065 bp    mRNA    linear
DEFINITION  Ceratitis capitata sub2-63 (Cc_sub2-63) cDNA, complete cds.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 2065)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2065)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
            Goettingen 37077, Germany
FEATURES             Location/Qualifiers
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LOCUS       Cc_sub2-65              1273 bp    mRNA    linear
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ACCESSION
VERSION
KEYWORDS    .
SOURCE      Ceratitis capitata
  ORGANISM  Ceratitis capitata
            Unclassified.
REFERENCE   1  (bases 1 to 1273)
  AUTHORS   Schetelig,M.F., Caceres,C., Zacharopoulou,A., Franz,G. and
            Wimmer,E.A.
  TITLE     Conditional embryonic lethality to improve the sterile insect
            technique in Ceratitis capitata (Diptera: Tephritidae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1273)
  AUTHORS   Schetelig,M.F. and Wimmer,E.A.
  TITLE     Direct Submission
  JOURNAL   Department of Developmental Biology,
            Goettingen Center for Molecular Biosciences, GZMB,
            Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology,
            Georg-August-University Goettingen, Justus-von-Liebig-Weg 11,
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                     TIRLRILDLLLRRDHLHERKIATNGRNTKIAFSTLDLSVDRAQNEKYSNLALRLLRKM
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```

7 Curriculum vitae

Dipl.-Biochem.
Marc Florian Schetelig
Department of Developmental Biology
Johann-Friedrich Blumenbach Institute of Zoology and Anthropology
Georg-August-University Göttingen, GZMB
Justus-von-Liebig-Weg 11
37077 Göttingen
Germany

Education

2004-2008	Phd at the Göttingen Centre for Molecular Biosciences (GZMB), Georg-August-University Göttingen, Germany supervised by Prof. Dr. Ernst A. Wimmer. Dissertation topic: „New molecular technologies to improve the Sterile Insect Technique for the Mediterranean fruitfly *Ceratitis capitata* (Diptera: Tephritidae)“
1999-2004	Diploma in Biochemistry at the University Bayreuth, Germany (Grade: sehr gut (A)). *Major exam subjects*: biochemistry, genetics, microbiology and biophysical chemistry. *Minor exam subject*: plant physiology. Diploma thesis: „Isolierung blastodermspezifisch exprimierter Gene aus der Mittelmeerfruchtfliege *Ceratitis capitata*“ (Grade: sehr gut (A)).
2003	Internship at the department for genetics (Prof. Dr. Christian Lehner, University Bayreuth, Germany) supervised by Dr. Ernst A. Wimmer. Topic: „Klonierung von *orthodenticle* aus der karibischen Fruchtfliege *Anastrepha suspensa*“.
2002	Internship at the department for plant physiology (Prof. Dr. Erwin Beck, University Bayreuth, Germany) supervised by Dr. Sebastian Fettig. Topic: „Transformation von *Arabidopsis thaliana* mit Cytokininoxidasen“.
1999	Internship at the „Institut für Lebensmittel-, Wasser- und Umweltanalytik Nürnberg GmbH“, Nürnberg, Germany.
1999	“Abitur” from German secondary school (Gymnasium Röthenbach a. d. Peg.; Germany)

Additional skills

Languages	German (mother-tongue), English (fluent written and spoken)
Computer	Extensive computer skills on Windows and Macintosh systems

Invited speaker

- Horizons in Molecular Biology 2007 - 4th International PhD Student Symposium (Göttingen, Germany, 09/15/2007).
- 3rd Robert-Bosch-Meeting for International Agricultural and Forestry Science 2006 (Kloster Seeon, Germany, 12/08/2006).
- 7th International Symposium of Fruit Flies of Economic Importance (Salvador de Bahia, Brazil, 09/13/2006).
- European PhD Course in Insect Science & Biotechnology (University Pavia, Italy, 06/14/2006).
- 2nd Robert-Bosch-Meeting for International Agricultural and Forestry Science 2005 (Kloster Seeon, Germany, 12/09/2005).

Awards and Grants

- Poster Price at the 4th International PhD Student Symposium "Horizons in Molecular Biology 2007", Göttingen, Germany, 09/15/2007.
- DAAD VIGONI 2007/08 exchange program.

Publications

Marc F. Schetelig, Carlos Caceres, Antigone Zacharopoulou, Gerald Franz & Ernst A. Wimmer. 2008. Conditional embryonic lethality to improve the sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae). Submitted.

Marc F. Schetelig◐, Bernhard G. M. Schmid◐, Grazyna Zimowska & Ernst A. Wimmer. 2008. Plasticity in mRNA expression and localization of *orthodenticle* within higher Diptera. Submitted.

Scolari, F.◐, **Schetelig, M.F.◐,** Bertin, S., Malacrida, A.R., Gasperi, G. & Wimmer, E.A. 2008. Fluorescent Sperm Marking to Improve the Fight against the Pest Insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae). *New Biotechnology* **25** (1): 76-84.

Marc F. Schetelig, Francesca Scolari, Alfred M. Handler, Giuliano Gasperi & Ernst A. Wimmer. 2008. New genetic tools for improving SIT in *Ceratitis capitata*: embryonic lethality and sperm marking. In *Proceedings, Symposium: 7th International Symposium on Fruit Flies of Economic Importance, 10-15 September 2006, Salvador, Bahia, Brazil.* Malavasi, A (ed.).

Schetelig, M.F., Horn, C., Handler, A.M. & Wimmer, E.A. 2007. Development of an embryonic lethality system for transgenic SIT in the fruit pest *Ceratitis capitata*. In *Area-Wide Control of Insect Pests: From Research to Field Implementation* (eds. Vreysen, M.J.B., Robinson A.S., Hendrichs, J.), pp 85-93. Dordrecht, NL: Springer.

◐ co-first author