Insect transgenesis applied to tephritid pest control

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Abstract

Tephritid fruit fly species cause major economical losses in crops worldwide. Genetic transformation of insect pests, which are targets of the Sterile Insect Technique (SIT), a key component of area-wide pest management, has been achieved for several years. For the medfly *Ceratitis capitata* as well as several *Bactrocera* and *Anastrepha* species, germline transformation can now be used to bioengineer strains that should increase the efficacy and cost-effectiveness of the SIT. Novel transformation vectors, robust genetic markers and diverse promoters to drive stage- and tissue-specific gene expression provide powerful tools to test the contribution that these technologies can make to current SIT programmes.

Introduction

The family Tephritidae (true fruit flies) encompasses more than 5000 species of phytophagous insects worldwide, approximately 1400 of which feed as larvae on fleshy fruits. The four major genera of this family, *Ceratitis*, *Bactrocera*, *Anastrepha* and *Rhagoletis* include pest species of remarkable economic importance (White and Elson-Harris 1992). Recently, members of these genera have been reported outside their respective home ranges as a result of the increasing fruit trade and the expansion of the tourist industry (Clarke et al. 2005; Malacrida et al. 2007). Their invasive potential is mainly because of the wide range of resource-exploitation strategies (Duyck et al. 2004), along with the high mobility, fecundity and the dispersive powers which characterize many tephritid species. During the last decades, researchers have concentrated their attention on the development and implementation of biological methods aimed at controlling pest populations, bypassing the traditional pesticide-based approaches.

The Sterile Insect Technique (SIT) is an environment-friendly method of pest control, which has become increasingly important in the context of area-wide integrated pest management (AW-IPM) programmes. Originally designed in the 1950s for the eradication of the New World screwworm in the USA (Knipling 1955), the SIT is currently applied to some major pests of plants and animals and human disease vectors. It has proven successful in reducing, controlling and eradicating economically important pest species such as the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Krafsur 1998). This technology involves the release of mass-reared and radiation-sterilized males into a wild population of the same species. The effectiveness of SIT relies on the mating competitiveness of the released sterile males, whose infertile matings lead to a decrease in the female reproductive potential and subsequently to a decline in population size.

Since its first application, the SIT has represented a major subject for research and development in the Joint FAO/IAEA Programme on Nuclear Techniques in Food and Agriculture, involving both research and the transfer of this technology to Member States, so that they can accelerate their economic development (Joint FAO/IAEA Division 1985). Besides the applications against medfly, SIT is currently used for the control of the Queensland fruit

fly, *Bactrocera tryoni* (Froggatt), which has been eradicated from Western Australia (Fisher 1994). Pilot SIT projects aimed at the eradication of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel), and the guava fruit fly, *Bactrocera correcta* (Bezzi), are underway in Thailand (Orankanok et al. 2007), while an eradication programme against *Bactrocera philippinensis* (Drew & Hancock) is ongoing in the Philippines (Covacha et al. 2000). The Mexican fruit fly, *Anastrepha ludens* (Loew), was eradicated from north-western Mexico (Reyes et al. 2000) and the melon fly, *Bactrocera cucurbitae* (Coquillett) from Japan (Koyama et al. 2004). In spite of its success and environmental benefits, the SIT has been used for only a few target species, because the sterilization by radiation and a series of technical aspects produce several negative loads on the treated males, resulting in reduced fitness and/or lifespan.

The development of genetic sexing strains (GSS) that permit the separation of males from females is crucial for an effective application of the SIT against tephritid pests (Robinson et al. 1999). By releasing only sterile males, the efficiency of the technique can be increased and fruit damage by sterile females that retain their oviposition behaviour is avoided (McInnis et al. 1994; Rendon et al. 2000). In order to obtain sex separation, GSS were established in the medfly using a classical genetic approach; these strains carry reciprocal translocations between the Y chromosome and an autosome carrying a recessive selectable marker (Franz 2005). Insecticide resistance, pupal colour (several pupal colour markers have been identified and used in various tephritid fruit flies; Robinson 2002a,b) and the medfly temperature-sensitive lethal (*tsl*) system are the most well-known selectable markers that have been used (Rendon et al. 2004; Franz 2005; fig. 2a-1). The *tsl* system is widely used and almost all sterile medfly-rearing facilities have converted to this strain. Nevertheless, these classical genetic systems cannot be transferred to other tephritid species by classical genetics. Moreover, all currently used GSS are based on Y-autosome translocations with the consequence that, due to adjacent-1 segregation during male meiosis, genetically unbalanced gametes are generated leading to significant levels of male sterility (Robinson et al. 1999), which impacts on the efficiency of mass rearing. The recent progress of gene transfer techniques is such that transgenic technology has now the potential to be transferred to a wide range of species. Furthermore, initial valuations of strains in field cage tests and large-scale rearing conditions are fundamental, especially in the light of the possible use of transgenic strains for field applications. In this framework, major technical problems concerning stability and safety of the transgenic strains and their ability to express the transgene in a reliable and predictable way have to be taken into account.

Here, we review the current state of the art of transgenic technology in tephritid species, focusing on the technical advances that are already available and those that will be necessary to improve further for: (i) the implementation of current pest control systems and (ii) widening our knowledge about several economically important pest species. Finally, as transgenic strains have already been generated for practical application, risk assessment issues for their use in laboratory studies and potential release in the field represent a top priority that needs to be addressed.

Available Tools for Genetic Transformation of Tephritids

Transposons and insect transformation

Currently, insect germline transformation is typically mediated by transposable elements (TEs). The first successful genomic manipulation was achieved in *Drosophila melanogaster*, taking advantage of the TE *P* (Rubin and Spradling 1982). Several trials were performed to transform pest insects by using *P*-element-based vectors, but they all failed because of the absence of specific host factors (Handler et al. 1993; O'Brochta and Atkinson 1996) and the lack of efficient transformation markers.

The genetic transformation of non-drosophilid insects using non-autonomous versions of TEs is now a routine procedure and mainly relies on five transposon-based systems: *mariner*, *Minos*, *Hermes*, *hobo* and *piggyBac* (Handler 2001; Handler and Harrell 2001a). The use of these elements allowed the transformation of several tephritid species, such as *C. capitata* (Loukeris et al. 1995; Handler et al. 1998; Michel et al. 2001), *B. dorsalis* (Handler and McCombs 2000), *Anastrepha suspensa* (Handler and Harrell 2001b), *A. ludens* (Condon et al. 2007) and *Bactrocera oleae* (Koukidou et al. 2006).

However, not all transposons have the same transformation efficiency. For example, *hobo* vectors have generally been limited to drosophilids (Handler 2001). On the contrary, *piggyBac*-based vectors have been demonstrated to be widely applicable for gene transfer in insects, enabling the transformation of a considerable number of insect species (Handler

2001, 2002). Similarly, the *Minos* vector is a highly mobile vector that works in a wide range of vertebrates and invertebrates and represents a powerful tool for functional genetic and genomic applications (Pavlopoulos et al. 2007).

General markers

To extend the use of these TE-based systems to a broad range of insect species, the availability of markers capable of allowing easy and clear identification of transgenic individuals represents a key point (Horn et al. 2002). Eye colour genes are useful tools for the detection of transformants in several species such as *C. capitata* and *B. dorsalis* (Zwiebel et al. 1995; Sarkar and Collins 2000). Fluorescent proteins like the enhanced green fluorescent protein (EGFP) were then used as transformation reporters as they are less affected by position effects because of the random integration of the transgene into the genome than the eye colour genes (Horn et al. 2002). Given that prolonged exposure to fluorescent light during screening may negatively interfere with the survival of the flies, a system that permits a fast and clear identification of transformants is fundamental. Therefore, the use of strong promoters has to be preferred. The constitutive promoter of the *polyubiquitin* (PUb) gene from *D. melanogaster* was successfully used to generate the transformation marker PUbnlsEGFP and to identify transformants not only of *D. melanogaster* (Handler and Harrell 1999), but also of *C. capitata* and *A. suspensa* (Handler and Harrell 2001b). Subsequently, the red fluorescent protein DsRed, and its variant DsRed1, driven by the *D. melanogaster* PUb promoter, produced brighter and more easily detectable fluorescence at lower magnifications than EGFP expression (Handler and Harrell 2001a). A universal transformation system, which allows the analysis of the same transgenic construct in different insect species, was generated using the artificial 3xP3 promoter, mediating fluorescent expression in larval, pupal and adult insect eyes (Berghammer et al. 1999; Horn and Wimmer 2000). Such a universal marker will make possible novel comparative studies within the field of insect biology.

Site-specific recombination as a tool for stabilizing transgenes

For the safe release of genetically modified insects into the field it is necessary to generate systems inert to any transposase source that could be present in the environment, as TEs belong to families of elements and cross-interactions may be possible. Therefore, a fundamental requirement is the development of non-autonomous transgenic insertions. A technique involving the excision of one transposon end in the genome of *D. melanogaster* transgenic flies represented a first basic step towards this goal (Handler et al. 2004) and a strategy based on the use of composite *piggyBac* elements is now available for post-integration removal of both transposon ends from TE-mediated insertions in medfly (Dafa'alla et al. 2006; fig. 1).

Another approach through which it might be possible to functionally modify the genomic situation after random transposon-based transformation is site-specific recombination. The different strategies aimed at inserting transgenes into a single position in the genome of *Drosophila* are based on site-specific recombinases capable of catalysing crossover between defined target sequences (Branda and Dymecki 2004). The most popular of these enzymes are FLP and Cre, which have been employed in numerous applications (Golic and Lindquist 1989; Golic 1991; Siegal and Hartl 1996, 2000; Heidmann and Lehner 2001); however, the efficiency of this

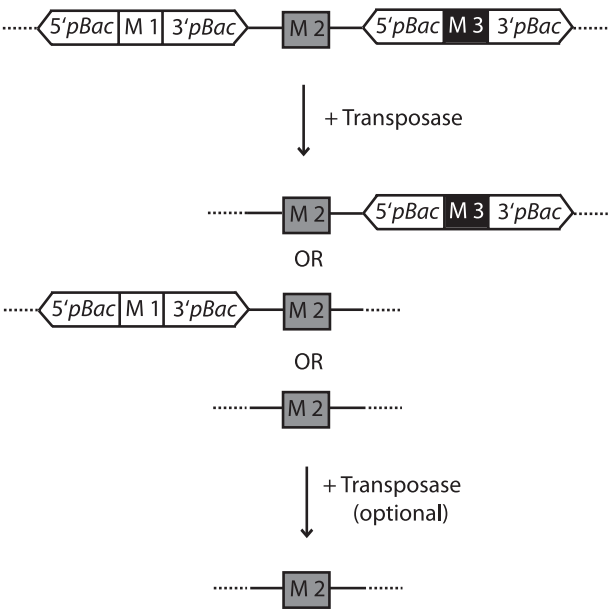

Fig. 1 Strategy available for post-integration removal of transposon ends from transposon-mediated insertions. The integrated transgene carries two pairs of opposed *piggyBac* transposon ends and three different markers (M1, M2 and M3). Exposure to the transposase results in the removal of the transposon(s) marked with M1 and/or M3, obtaining a stabilized transgenic insertion marked with M2. A further exposure to transposase might be required to remove both flanking transposons (adapted from Dafa'alla et al. 2006).

method is limited by its reversible nature (Golic et al. 1997). Another system that overcomes this limitation employs the integrase from the phage *ϕC31* (Thorpe and Smith 1998; Groth et al. 2004), which mediates unidirectional recombination between two sequences: the phage attachment site (*attP*) and the bacterial attachment site (*attB*). Their recombination generates two new hybrid sequences, *attR* and *attL* (Kuhstoss and Rao 1991; Rausch and Lehmann 1991), which no longer represent substrates for integrase (Thorpe et al. 2000). This strategy has been proven to be highly efficient in *Drosophila* (Groth et al. 2004) and even large DNA fragments up to 140 kb could be inserted (Venken et al. 2006). In the future it might be possible to take advantage of site-specific integration systems to perform functional studies at characterized genomic positions in tephritids and rearrange, delete or insert transgenic systems at favourable positions within the genome (Wimmer 2005a).

The ability to remove the transposon ends is of major importance, because it still needs to be clarified whether the presence of potentially active TEs could represent a risk for transgenesis applications. First, transposase produced by endogenous elements may destabilize integrated heterologous elements and transgenes (Sundararajan et al. 1999). The potential risk of cross-mobilization or non-homologous recombination resulting in the instability of transgenes integrated into the genome have to be carefully evaluated. For example, there is evidence that the *hobo* element from *D. melanogaster* and the *Hermes* element from *Musca domestica* can interact in such a way as to cause vector destabilization (Sundararajan et al. 1999). The isolation of a potentially active *hobo*-like sequence in the medfly (*Cchobo*, Torti et al. 2005) and the transformation of the medfly using *Hermes* (Michel et al. 2001) raise questions about the possibility of *Cchobo*/*Hermes* reciprocal cross-mobilization and about the stability of such transformation systems.

Secondly, the movements of endogenous TEs might result in mutations or reduced viability. In fact, several factors within the host genome might interact with the transgene vector causing its remobilization or inhibiting its movements, preventing, for example, its spread through a population (Sundararajan et al. 1999). Horizontal transfer of TEs has been well documented (Kidwell 1992a,b; Simmons 1992; Robertson and Lampe 1995; Jordan et al. 1999). Because genetic barriers between species are not completely impenetrable to gene flow, the ability of a gene vector system to function also in non-target organisms is of great ecological concern (Atkinson et al. 2001; Handler 2002). Therefore, the potential of transgene remobilization has to be assessed and represents a priority for risk assessment.

Finally, transformation efficiency can be negatively influenced by the interaction between the transposase from an endogenous TE, with the transposase associated with the vector system resulting in the blocking of binding sites, or through the formation of non-functional transposase multimers.

To date, the medfly genome has been shown to contain several TE from the *mariner*, *Tc1*, *hAT* and *gypsy/Ty3* families (Handler and Gomez 1996; Zhou and Haymer 1998; Gomulski et al. 2004; Torti et al. 2005) displaying different levels of diversity, abundance and distribution in the genome. The presence of actively transposing elements in the medfly genome is revealed by hybrid dysgenesis phenomena, insertion site polymorphisms and other genetic instabilities (Torti et al. 1997).

As *piggyBac*-based systems are widely used for transformation of tephritids, precise knowledge of the host distribution of the *piggyBac* element has important implications for transgene stability and horizontal transmission. The presence of endogenous *piggyBac*-like elements in *B. dorsalis* s.s. has to raise caution on the stability of the transgene, even if integrations have been shown to remain stable for more than 20 generations in flies transformed using a *piggyBac*-based vector system (Handler 2002). The absence of *piggyBac*-like sequences in other tephritid species, such as *C. capitata*, *Anastrepha* spp. and *B. oleae*, is promising for the successful application of such vectors (Bonizzoni et al. 2007).

In this background, it follows that exploration of the genomes of tephritids will have a strong impact on our knowledge of their biology, allowing the development of novel tools to improve pest control methods. For example, among the medfly expressed sequence tags (ESTs), a total of 63 transcripts showed significant homology to known TEs (Gomulski et al. 2008). The medfly has been a target of transformation studies involving the exogenous elements *Minos*, *Hermes* and *piggyBac* (Loukeris et al. 1995; Handler et al. 1998; Michel et al. 2001) and the presence of active endogenous homologous elements can have important implications for the stability of such transgenic lines. It is therefore evident that functional genomics is gaining more and more importance also in terms of improving control programmes. In addition, transgenic approaches might allow us to functionally characterize the sequences identified in genome projects, permitting direct

correlation of sequence data with biological function (Wimmer 2003).

Transgenesis and its Impact on Pest Control

Transgenic technology may enhance operational SIT programmes at three levels: genetic sexing, sterilization and monitoring. First, transgenesis can optimize male-only production through genetic sexing strategies (fig. 2a-2). Molecular approaches can be used to transform females into males as achieved by RNAi against the *C. capitata transformer* gene (Pane et al. 2002), or to eliminate females. The release of insects carrying a dominant lethal (RIDL) is a strategy that combines both genetic sexing and 'sterilization' from the same construct (Alphey 2002; Gong et al. 2005; Wimmer 2005b). Transgene-based genetic sexing mechanisms (GSMs) using conditional female-specific lethality systems, based on the tetracycline-repressible expression system of Gossen and Bujard (1992), were successfully tested in *Drosophila* (Heinrich and Scott 2000; Thomas et al. 2000). Medfly strains expressing a tetracycline-repressible transactivator (tTA) that causes lethality in late developmental stages of heterozygous progeny have been produced (Gong et al. 2005; fig. 2b-1). Recently, medfly embryonic genes have been isolated to transfer the principle of reproductive sterility based on embryonic lethality (Horn and Wimmer 2003) to tephritid pest species (Schetelig et al. 2007, 2008; fig. 2b-2). Sex-specific alternative splicing to engineer female-specific autocidal genetic systems was also exploited in the medfly (Fu et al. 2007) and demonstrated the potential of controlling gene expression specifically in female embryos.

The release of transgenically marked sterile males as part of ongoing SIT programmes might have a role in short-term releases for agricultural pest suppression. As the presence of transgenes in the environment is limited to the sterilized flies carrying harmless fluorescent protein-based markers, there will be no vertical transmission, thus favouring their use in field test feasibility studies. The introduction of transgenic strains may also be useful in improving the effectiveness of monitoring procedures, overcoming the disadvantages of the present systems. To date, sterilized insects reared in laboratories are marked at the pupal stage with fluorescent powders, allowing them to be distinguished from wild flies when recaptured in traps within the release area. Although dust marking is the most commonly used system, dusts have been reported to inhibit normal dispersal behaviour (Chang 1946), to decrease insect longevity (Sheppard et al. 1969; Reinecke 1990; Messing and Seiler 1993), to be non-persistent for long-term studies, expensive, difficult to handle, dangerous for human health and error-prone (Hagler and Jackson 2001). Moreover, fluorescent powder usage represents an additional step during mass rearing. Therefore, it would represent a great improvement to have a marker that (i) is retained on the insect for a sufficient period of time (corresponding to the interval between two successive trap controls); (ii) does not interfere with normal insect behaviour, growth, reproduction or life span; (iii) is inexpensive; (iv) is non-toxic to insect and environment; (v) is easy to handle; and (vi) is clearly identifiable (Hagler and Jackson 2001).

Specific markers

The availability of sex-specific fluorescent markers represents a powerful tool for the improvement of the monitoring procedures, because such markers have all the necessary features for practical use in the field. Moreover, if expressed early enough, they can be useful for sexing, as already proven for the mosquitoes *Anopheles stephensi* (Catteruccia et al. 2005) and *Aedes aegypti* (Smith et al. 2007).

In Scolari et al. (2008), two testes-specific markers for *C. capitata* were generated by fusing the promoter of the spermatogenesis-specific medfly *β2-tubulin* gene with the reporter genes encoding a red or green fluorescent protein, respectively (fig. 3a). Besides improving SIT monitoring, these markers will enable studies of medfly mating behaviour at several levels, such as sperm transfer, sperm storage, sperm use, sperm precedence and sperm competition (Bonizzoni et al. 2006; Yuval et al. 1996; fig. 3b). A preliminary set of laboratory competitiveness tests on these testis-specific marked flies was performed to estimate the mating ability of transgenic homozygous males. The copulation latency of transgenic and wild-type (WT) males was recorded to check whether transgenic males differed from WT males in the time required to gain copulations. As an additional assessment of the overall fitness of the testes-marked homozygous medfly lines, the effect of transgenic marking on the reproductive capacity and the proportion of progeny sired by transgenic or WT males in competitive conditions were tested. As previously stated, the efficiency of a transformation event depends not only on the chosen vector, but also on the markers used to verify the transformation event itself. In both systems, a double marking approach was followed, in which the PUb promoter

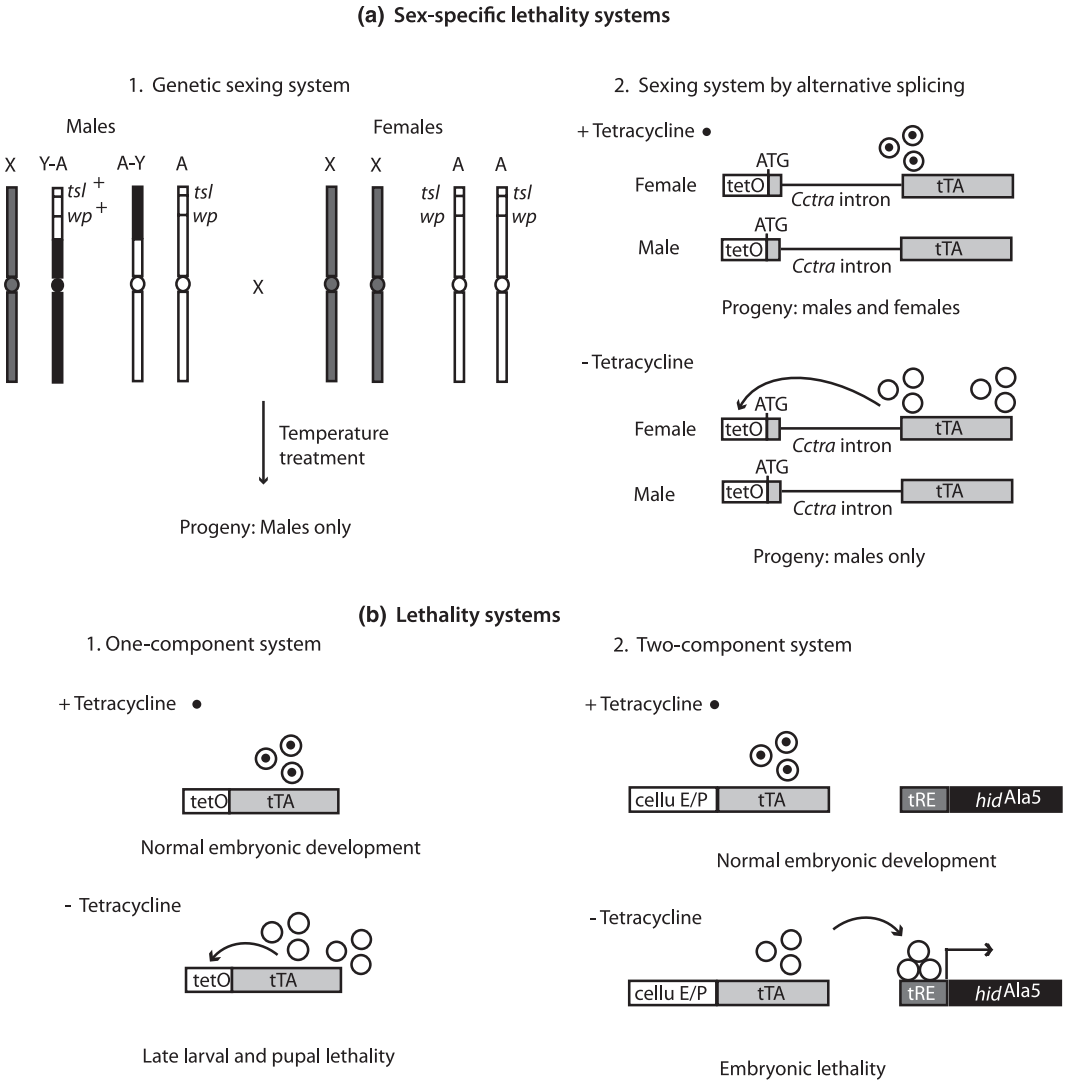

Fig. 2 Transgenic approaches to improve the SIT. (a) Sex-specific lethality systems. (1) The *tsl* system aims to produce only male progeny: it relies on a translocation on the Y chromosome carrying a dominant *tsl*$^{+}$ gene that causes male resistance to heat treatment (adapted from Franz 2005). (2) Female-specific lethality system which combines the principle of RIDL and the properties of the *Cctra* gene intron. The dominant lethal gene encodes for the tetracycline-repressible transcription factor (tTA) which is lethal at high concentrations and is inactivated by the presence of tetracycline. The tTA expression is under the control of the tetracycline operator (tetO), which, in the absence of tetracycline, is bound and activated by the tTA protein itself. An engineered tTA gene, whose coding sequence is interrupted by the insertion of the *Cctra* intron, expresses a full-length protein only in females, because the *Cctra* intron which carries STOP codons is maintained in males but spliced in females. Therefore, in the absence of tetracycline, the concentration of the tTA causes female-specific lethality (adapted from Fu et al. 2007). (b) Lethality systems. (1) In the one-component system, the RIDL basic principles are applied to obtain larval, pupal and/or adult lethality, irrespective of the sex of embryos (adapted from Gong et al. 2005). (2) The two-component system represents an embryonic lethality system based on the expression of a dominant lethal gene (*hid*^{Ala5}) during the first steps of embryogenesis. In embryos, in absence of tetracycline, tTA, whose expression is under the control of cellu E/P (enhancer–promoters of cellularization genes), binds the tRE (tetracycline-responder element) and induces the expression of the dominant lethal gene (adapted from Schetelig et al. 2007).

of *D. melanogaster* drives the expression of EGFP or DsRed and the newly isolated *β2-tubulin* putative promoter drives the expression of tGFP (turboGFP) or DsRedEx (DsRedExpress), respectively. The efficiency of the *piggyBac* transformation system together with fluorescent markers permitted the selection of many transgenic lines and represented the fundamental background for *in vivo* verification of the expression of the medfly *β2-tubulin* putative promoter. Stable marking in testes and sperm

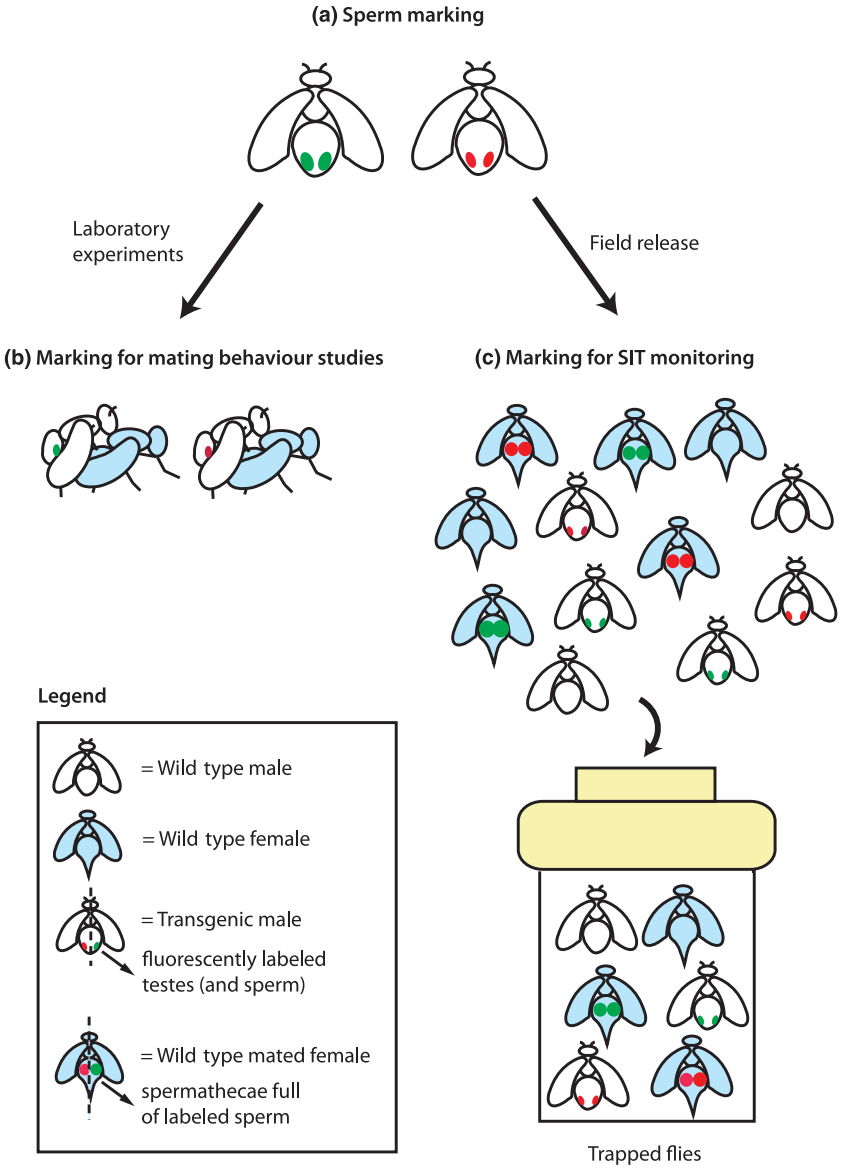

Fig. 3 Fluorescent sperm marking as a tool for the improvement of medfly mating behaviour studies and SIT monitoring. (a) Transgenic males with green or red fluorescent testes. (b) The availability of transgenic males with fluorescent testes and sperm will allow novel studies on reproductive biology related to the mating behaviour of this polyandrous species. (c) The field release of such transgenic sterilized males will enable the assessment of the efficacy of SIT programmes; data collected from traps located within the release area will give information on the number and mating success of sterilized males and on the mating status of WT females.

depends on the availability of specific promoters and of intensely fluorescent molecules. As in *D. melanogaster*, the medfly *β2-tubulin* promoter was shown to control gene expression at testes level and during spermatogenesis. This promoter is also able to direct the expression of DsRedEx and tGFP, which permit clear marking of the testes and single sperm. The *β2t*-DsRedEx/tGFP vector provides a selective and potent marking system for the analysis of medfly reproductive biology.

DsRed and DsRedEx expression were detectable in adult individuals up to 3 months after death; tGFP showed a comparable longlife, while the EGFP body marker lost its intensity after 2–3 days in live flies. These results are particularly important for the use of such transgenic flies in SIT monitoring procedures, because it will be possible to detect fluorescence in flies captured in traps long after their death. In addition, sperm marking will facilitate the assessment of the mating status of trapped females, which in turn, will allow the mating efficiency of the released sterile males to be monitored (fig. 3c).

Sterile male fitness assessment for successful SIT programmes

As successful applications of SIT programmes depend on the mating competitiveness of the transgenic males, the transgenes should impose a minimal fitness load on their hosts. Fitness assessment studies

are of major importance because they will permit the selection of competitive genetically modified insects for use in control programmes (Irvin et al. 2004). Fitness, which can be defined as the relative success with which a genotype transmits its genes to the next generation, encompasses different components such as survival, reproduction and development (Marrelli et al. 2006). Therefore, several parameters have to be analysed: fertility, fecundity, larval biomass productivity, developmental rate, adult emergence, sex ratio, mating competitiveness, etc.

Transgenic technologies may impact fitness for two main reasons: (i) fluorescent proteins which act as the transformation marker might accumulate in large amounts causing a fitness load (Liu et al. 1999); (ii) as transformation through microinjection into the germline occurs randomly, the transgene might get inserted in transcriptionally active areas of the genome (Spradling et al. 1995, 1999; Thibault et al. 2004). In order to limit the negative effects caused by insertional mutagenesis, it is essential to generate multiple transgenic lines, compare them and select the fittest (Marrelli et al. 2006). Moreover, the strength of transgene expression can be influenced by chromatin surrounding the insertion site (position effect); when the strength of expression is important, the availability of several independently obtained lines of the same construct is fundamental. Alternatively, the use of insulator elements could be considered (Sarkar et al. 2006).

The measurement of fitness is difficult as it depends on numerous environmental or genetic variables. Furthermore, when testing fitness in the laboratory, it is important that the experimental-design allows male–male competition, permitting direct comparison between transgenic and wild-type insects. Mating success is determined by both intersexual and intrasexual selection between males in the limited space inside the cages. Intrasexual selection may involve aggressive interactions which reduce the time available to interact with the female, directly influencing the final fitness result (Calcagno et al. 2002). Moreover, laboratory rearing conditions are characterized by a dramatic reduction of space and absence of natural constraints (lek formation, fruits, daylight, etc.). Mass rearing conditions probably favour shortened courtship and fast mating (Calcagno et al. 1999), and most probably increased male aggressiveness.

For all these reasons, three phases of cage competition experiments are needed: first of all, a series of tests performed in the laboratory; then it is important to perform competition experiments between transgenic and endemic, locally sampled, wild-type flies within cages at the proposed release site; finally large field enclosures tests are necessary (Scott et al. 2005).

Conclusions

In conclusion, the proposed transgenic applications demonstrate two additional advances besides the discussed improvement of the SIT: (i) ecological insights into the impact of transgenic insects in the natural environment; (ii) support for discussion of the safe use of biotechnology in insect pest control. Transgenes should be used critically, always taking into account that risk assessment surveys are essential for any field application. This is particularly important for insects, which have enormous reproductive and dispersal potentials. Furthermore, releases of transgenic insects are likely to involve large areas of land and it will be extremely difficult to control or eradicate transgenic strains after their release into the environment (Atkinson et al. 2001).

Currently medfly SIT applications are based on two major components: (a) male-only production and release through the use of GSS and (b) the use of radiation for sterilizing the males. The medfly SIT programmes could therefore be a good starting point for implementing and testing transgenic technology, especially given their efficient quality control systems which will ensure their safe use.

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References

Alphey L, 2002. Re-engineering the sterile insect technique. Insect Biochem. Mol. Biol. 32, 1243–1247.

Atkinson PW, Pinkerton AC, O'Brochta DA, 2001. Genetic transformation systems in insects. Annu. Rev. Entomol. 46, 317–346.

Berghammer AJ, Klinger M, Wimmer EA, 1999. A universal marker for transgenic insects. Nature 402, 370–371.

Bonizzoni M, Gomulski LM, Mossinson S, Guglielmino CR, Malacrida AR, Yuval B, Gasperi G, 2006. Is polyandry a common event among wild populations of

the pest *Ceratitis capitata*? J. Econ. Entomol. 99, 1420–1429.

Bonizzoni M, Gomulski LM, Malacrida AR, Capy P, Gasperi G, 2007. Highly similar *piggyBac* transposase-like sequences in various *Bactrocera* (Diptera, Tephritidae) species. Insect Mol. Biol. 16, 645–650.

Branda CS, Dymecki SM, 2004. Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice. Dev. Cell 6, 7–28.

Calcagno G, Vera MT, Manso F, Lux S, Norry F, Munyiri N, Vilardi JC, 1999. Courtship behavior of wild and mass-reared Mediterranean fruit fly (Diptera: Tephritidae) males from Argentina. J. Econ. Entomol. 92, 373–379.

Calcagno GE, Manso F, Vilardi JC, 2002. Comparison of mating performances for genetic sexing and wild type strains of *Ceratitis capitata* (Diptera: Tephritidae): field cage and video recording experiments. Fl. Entomol. 85, 41–50.

Catteruccia F, Benton JP, Crisanti A, 2005. An *Anopheles* transgenic sexing strain for vector control. Nat. Biotechnol. 23, 1414–1417.

Chang HT, 1946. Studies on the use of fluorescent dyes for marking *Anopheles quadrimaculatus*. Mosq. News 6, 122–125.

Clarke AR, Armstrong KF, Carmichael AE, Milne JR, Raghu S, Roderick GK, Yeates DK, 2005. Invasive phytophagous pest arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. Annu. Rev. Entomol. 50, 293–319.

Condon KC, Condon GC, Dafa'alla TH, Forrester OT, Phillips CE, Scaife S, Alphey L, 2007. Germ-line transformation of the Mexican fruit fly. Insect Mol. Biol. 16, 573–580.

Covacha SA, Bignayan HG, Gaitan EG, Zamora NF, Maranon RP, Manoto EC, Obra GB, Resilva SS, Reyes MR, 2000. Status report on ''the integrated fruit fly management based on the sterile insect technique in Guimaras Island, Philippines''. In: Area-wide control of fruit flies and other insect pests. Ed. by Tan KH, Penerbit Universiti Sains Malaysia, Penang, 401–408.

Dafa'alla TH, Condon GC, Condon KC, Phillips CE, Morrison NI, Jin L, Epton MJ, Fu G, Alphey L, 2006. Transposon-free insertions for insect genetic engineering. Nat. Biotechnol. 24, 820–821.

Duyck PF, David P, Quilici S, 2004. A review of relationships between interspecific competition and invasions in fruit flies (Diptera: Tephritidae). Ecol. Entomol. 29, 511–520.

Fisher KT, 1994. Eradication of Queensland Fruit Fly, *Bactrocera tryoni*, from Western Australia. In: Fruit flies and the sterile insect technique. Ed by Calkins CO, Klassen W, Liedo P, CRC Press, Boca Raton, FL, 237–246.

Franz G, 2005. Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. In: Sterile insect technique. Principles and practice in area-wide integrated pest management. Ed by Dyck VA, Hendrichs J, Robinson AS, Springer, Dordrecht, 427–451.

Fu G, Condon KC, Epton MJ, Gong P, Jin L, Condon GC, Morrison NI, Dafa'alla TH, Alphey L, 2007. Female-specific insect lethality engineered using alternative splicing. Nat. Biotechnol. 25, 353–357.

Golic KG, 1991. Site-specific recombination between homologous chromosomes in *Drosophila*. Science 252, 958–961.

Golic KG, Lindquist S, 1989. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. Cell 59, 499–509.

Golic MM, Rong YS, Petersen RB, Lindquist SL, Golic KG, 1997. FLP-mediated DNA mobilization to specific target sites in *Drosophila* chromosomes. Nucleic Acids Res. 25, 3665–3671.

Gomulski LM, Torti C, Murelli V, Bonizzoni M, Gasperi G, Malacrida AR, 2004. Medfly transposable elements: diversity, evolution, genomic impact and possible applications. Insect Biochem. Mol. Biol. 34, 139–148.

Gomulski LM, Dimopoulos G, Xi Z, Soares MB, Bonaldo MF, Malacrida AR, Gasperi G, 2008. Gene discovery in an invasive tephritid model pest species, the Mediterranean fruit fly, *Ceratitis capitata*. BMC Genomics 9, 243.

Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC, Condon GC, Morrison NI, Kelly DW, Dafa'alla TH, Coleman PG, Alphey L, 2005. A dominant lethal genetic system for autocidal control of the Mediterranean fruit fly. Nat. Biotechnol. 23, 453–456.

Gossen M, Bujard H, 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl Acad. Sci. USA 89, 5547–5551.

Groth AC, Fish M, Nusse R, Calos MP, 2004. Construction of transgenic *Drosophila* by using the site-specific integrase from phage *phiC31*. Genetics 166, 1775–1782.

Hagler JR, Jackson CG, 2001. Methods for marking insects: current techniques and future prospects. Annu. Rev. Entomol. 46, 511–543.

Handler AM, 2001. A current perspective on insect gene transformation. Insect Biochem. Mol. Biol. 31, 111–128.

Handler AM, 2002. Use of the *piggyBac* transposon for germ-line transformation of insects. Insect Biochem. Mol. Biol. 32, 1211–1220.

Handler AM, Gomez SP, 1996. The *hobo* transposable element excises and has related elements in tephritid species. Genetics 143, 1339–1347.

Handler AM, Harrell RA, 1999. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Mol. Biol. 8, 449–457.

Handler AM, Harrell RA II, 2001a. Polyubiquitin-regulated DsRed marker for transgenic insects. Biotechniques 31, 824–828.

Handler AM, Harrell RA II, 2001b. Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a *piggyBac* vector marked with polyubiquitin-regulated GFP. Insect Biochem. Mol. Biol. 31, 199–205.

Handler AM, McCombs SD, 2000. The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. Insect Mol. Biol. 9, 605–612.

Handler AM, Gomez SP, O'Brochta DA, 1993. A functional analysis of the *P*-element gene-transfer vector in insects. Arch. Insect Biochem. Physiol. 22, 373–384.

Handler AM, McCombs SD, Fraser MJ, Saul SH, 1998. The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the Mediterranean fruit fly. Proc. Natl Acad. Sci. USA 95, 7520–7525.

Handler AM, Zimowska GJ, Horn C, 2004. Post-integration stabilization of a transposon vector by terminal sequence deletion in *Drosophila melanogaster*. Nat. Biotechnol. 22, 1150–1154.

Heidmann D, Lehner CF, 2001. Reduction of Cre recombinase toxicity in proliferating *Drosophila* cells by estrogen-dependent activity regulation. Dev. Genes. Evol. 211, 458–465.

Heinrich JC, Scott MJ, 2000. A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. Proc. Natl Acad. Sci. USA 97, 8229–8232.

Horn C, Wimmer EA, 2000. A versatile vector set for animal transgenesis. Dev. Genes. Evol. 210, 630–637.

Horn C, Wimmer EA, 2003. A transgene-based embryo-specific lethality system for insect pest management. Nat. Biotechnol. 21, 64–70.

Horn C, Schmid BG, Pogoda FS, Wimmer EA, 2002. Fluorescent transformation markers for insect transgenesis. Insect Biochem. Mol. Biol. 32, 1221–1235.

Irvin N, Hoddle MS, O'Brochta DA, Carey B, Atkinson PW, 2004. Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. Proc. Natl Acad. Sci. USA 101, 891–896.

Joint FAO/IAEA Division, 1985. Report of the Consultants' Meeting on: The application of genetic engineering and recombinant DNA technology in the development of genetic sexing mechanisms for the Mediterranean fruit fly, Ceratitis capitata (Wiedemann). International Atomic Energy Agency, Vienna.

Jordan IK, Matyunina LV, McDonald JF, 1999. Evidence for the recent horizontal transfer of long terminal repeat retrotransposon. Proc. Natl Acad. Sci. USA 96, 12621–12625.

Kidwell MG, 1992a. Horizontal transfer. Curr. Opin. Genet. Dev. 2, 868–873.

Kidwell MG, 1992b. Horizontal transfer of *P* elements and other short inverted repeat transposons. Genetica 86, 275–286.

Knipling EF, 1955. Possibilities of insect control or eradication through the use of sexually sterile males. J. Econ. Entomol. 48, 459–462.

Koukidou M, Klinakis A, Reboulakis C, Zagoraiou L, Tavernarakis N, Livadaras I, Economopoulos A, Savakis C, 2006. Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker. Insect Mol. Biol. 15, 95–103.

Koyama J, Kakinohana H, Miyatake T, 2004. Eradication of the melon fly, *Bactrocera cucurbitae*, in Japan: importance of behavior, ecology, genetics, and evolution. Annu. Rev. Entomol. 49, 331–349.

Krafsur ES, 1998. Sterile insect technique for suppressing and eradicating insect populations: 55 years and counting. J. Agric. Entomol. 15, 303–317.

Kuhstoss S, Rao RN, 1991. Analysis of the integration function of the streptomycete bacteriophage *phiC31*. J. Mol. Biol. 222, 897–908.

Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ, 1999. Is green fluorescent protein toxic to the living cells? Biochem. Biophys. Res. Commun. 260, 712–717.

Loukeris TG, Livadaras I, Arcà B, Zabalou S, Savakis C, 1995. Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. Science 270, 2002–2005.

Malacrida AR, Gomulski LM, Bonizzoni M, Bertin S, Gasperi G, Guglielmino CR, 2007. Globalization and fruit-fly invasion and expansion: the medfly paradigm. Genetica 131, 1–9.

Marrelli MT, Moreira CK, Kelley D, Alphey L, Jacobs-Lorena M, 2006. Mosquito transgenesis: what is the fitness cost? Trends Parasitol. 22, 197–202.

McInnis DO, Wong TTY, Nishimoto J, 1994. Population suppression and sterility rates induced by variable sex ratio, sterile insect releases of *Ceratitis capitata* (Diptera: Tephritidae) in Hawaii. Ann. Ent. Soc. Am. 87, 231–240.

Messing RH, Seiler SJ, 1993. Malathion leakage from fruit fly male-annihilation traps on Kauai, Hawaii. Bull. Environ. Contam. Toxicol. 51, 193–198.

Michel K, Stamenova A, Pinkerton AC, Franz G, Robinson AS, Gariou-Papalexiou A, Zacharopoulou A, O'Brochta DA, Atkinson PW, 2001. *Hermes*-mediated germ-line transformation of the Mediterranean fruit fly *Ceratitis capitata*. Insect Mol. Biol. 10, 155–162.

O'Brochta DA, Atkinson PW, 1996. Transposable elements and gene transformation in non-Drosophilid insects. Insect Biochem. Mol. Biol. 26, 739–753.

Orankanok W, Chinvinijkul S, Thanaphum S, Sitilob P, Enkerlin WR, 2007. Area-wide integrated control of oriental fruit fly *Bactrocera dorsalis* and guava fruit fly *Bactrocera correcta* in Thailand. In: Area-wide control of

insect pests: from research to field implementation. Ed by Vreysen MJB, Robinson AS, Hendrichs J, Springer, Dordrecht, 517–526.

Pane A, Salvemini M, Delli Bovi P, Polito C, Saccone G, 2002. The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. Development 129, 3715–3725.

Pavlopoulos A, Oehler S, Kapetanaki MG, Savakis C, 2007. The DNA transposon *Minos* as a tool for transgenesis and functional genomic analysis in vertebrates and invertebrates. Genome Biol. 8(Suppl. 1), S2.

Rausch H, Lehmann M, 1991. Structural analysis of the actinophage *phiC31* attachment site. Nucleic Acids Res. 19, 5187–5189.

Reinecke JP, 1990. A rapid and controllable technique for surface labeling boll weevils with fluorescent pigments. South-west Entomol. 15, 309–316.

Rendon P, McInnis DO, Lance DL, Stewart J, 2000. Comparison of medfly male-only and bisex releases in large-scale field trials. In: Area-wide control of fruit flies and other insect pests. Ed. by Tan KH, Penerbit Universiti Sains Malaysia, Penang, 517–526.

Rendon P, McInnis D, Lance D, Stewart J, 2004. Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. J. Econ. Entomol. 97, 1547–1553.

Reyes J, Santiago G, Hernandez P, 2000. The Mexican fruit fly eradication programme. In: Area-wide control of fruit flies and other insect pests. Ed. by Tan KH, Penerbit Universiti Sains Malaysia, Penang, 377–380.

Robertson HM, Lampe DJ, 1995. Recent horizontal transfer of a *mariner* transposable element among and between Diptera and Neuroptera. Mol. Biol. Evol. 12, 850–862.

Robinson AS, 2002a. Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes. Genetica 116, 5–13.

Robinson AS, 2002b. Mutations and their use in insect control. Mutat. Res. 511, 113–132.

Robinson AS, Franz G, Fisher K, 1999. Genetic sexing strains in the medfly, *Ceratitis capitata*: development, mass rearing and field application. Trends Entomol. 2, 81–104.

Rubin GM, Spradling AC, 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218, 348–353.

Sarkar A, Collins FH, 2000. Eye color genes for selection of transgenic insects. In: Insect transgenesis: methods and applications. Ed by Handler AM, James AA, CRC Press, Boca Raton, FL, 79–93.

Sarkar A, Atapattu A, Belikoff EJ, Heinrich JC, Li X, Horn C, Wimmer EA, Scott MJ, 2006. Insulated *piggyBac* vectors for insect transgenesis. BMC Biotechnol. 6, 27.

Schetelig MF, Horn C, Handler AM, Wimmer EA, 2007. Development of an embryonic lethality system in Mediterranean fruit fly *Ceratitis capitata*. In: Area-wide control of insect pests: from research to field implementation. Ed by Vreysen MJB, Robinson AS, Hendrichs J, Springer, Dordrecht, 85–93.

Schetelig MF, Scolari F, Handler AM, Gasperi G, Wimmer EA, 2008. New genetic tools for improving SIT in *Ceratitis capitata*: embryonic lethality and sperm marking. In: Proceedings of the 7th International Symposium on fruit flies of economic importance, Salvador, Bahia, Brazil, 2006. Ed. by Malavasi A, ADAB–Agencia de Defesa Agropecuaria da Bahia, Salvador, Bahia, Brazil, 1–19.

Scolari F, Schetelig MF, Bertin B, Malacrida AR, Gasperi G, Wimmer EA, 2008. Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae). Nat. Biotechnol. 25, 76–84.

Scott TW, Rasgon JL, Black WC IV, Gould F, 2005. Fitness studies: developing a consensus methodology. In: Strategic plan to bridge laboratory and field research in disease vector control. Ed by Knols BGJ, Louis C, Frontis, Dordrecht, 171–181.

Sheppard PM, Macdonald WW, Tonn RJ, 1969. A new method of measuring the relative prevalence of *Aedes aegypti*. Bull. WHO 40, 467–468.

Siegal ML, Hartl DL, 1996. Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*. Genetics 144, 715–726.

Siegal ML, Hartl DL, 2000. Application of Cre/loxP in *Drosophila.* Site-specific recombination and transgene coplacement. Methods Mol. Biol. 136, 487–495.

Simmons GM, 1992. Horizontal transfer of *hobo* transposable elements within the *Drosophila melanogaster* species complex: evidence from DNA sequencing. Mol. Biol. Evol. 9, 1050–1060.

Smith RC, Walter MF, Hice RH, O'Brochta DA, Atkinson PW, 2007. Testis-specific expression of the beta2 tubulin promoter of *Aedes aegypti* and its application as a genetic sex-separation marker. Insect Mol. Biol. 16, 61–71.

Spradling AC, Stern DM, Kiss I, Roote J, Laverty T, Rubin GM, 1995. Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. Proc. Natl Acad. Sci. USA 92, 10824–10830.

Spradling AC, Stern D, Beaton A, Rhem EJ, Laverty T, Mozden N, Misra S, Rubin GM, 1999. The Berkeley *Drosophila* Genome Project gene disruption project: single *P*-element insertions mutating 25% of vital *Drosophila* genes. Genetics 153, 135–177.

Sundararajan P, Atkinson PW, O'Brochta DA, 1999. Transposable element interactions in insects:

crossmobilization of *hobo* and *Hermes*. Insect Mol. Biol. 8, 359–368.
Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, Singh CM, Buchholz R, Demsky M, Fawcett R, Francis-Lang HL, Ryner L, Cheung LM, Chong A, Erickson C, Fisher WW, Greer K, Hartouni SR, Howie E, Jakkula L, Joo D, Killpack K, Laufer A, Mazzotta J, Smith RD, Stevens LM, Stuber C, Tan LR, Ventura R, Woo A, Zakrajsek I, Zhao L, Chen F, Swimmer C, Kopczynski C, Duyk G, Winberg ML, Margolis J, 2004. A complementary transposon tool kit for *Drosophila melanogaster* using *P* and *piggyBac*. Nat. Genet. 36, 283–287.
Thomas DD, Donnelly CA, Wood RJ, Alphey LS, 2000. Insect population control using a dominant, repressible, lethal genetic system. Science 287, 2474–2476.
Thorpe HM, Smith MC, 1998. In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc. Natl Acad. Sci. USA 95, 5505–5510.
Thorpe HM, Wilson SE, Smith MC, 2000. Control of directionality in the site-specific recombination system of the *Streptomyces* phage *phiC31*. Mol. Microbiol. 38, 232–241.
Torti C, Gomulski LM, Malacrida AR, Capy P, Gasperi G, 1997. Genetic and molecular investigations on the endogenous mobile elements of non-drosophilid fruitflies. Genetica 100, 119–129.
Torti C, Gomulski LM, Bonizzoni M, Murelli V, Moralli D, Guglielmino CR, Raimondi E, Crisafulli D, Capy P, Gasperi G, Malacrida AR, 2005. *Cchobo*, a *hobo*-related sequence in *Ceratitis capitata*. Genetica 123, 313–325.
Venken KJ, He Y, Hoskins RA, Bellen HJ, 2006. P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. Science 314, 1747–1751.
White IM, Elson-Harris MM, 1992. Fruit flies of economic significance; their identification and bionomics. CAB International, Wallingford.
Wimmer EA, 2003. Innovations: applications of insect transgenesis. Nat. Rev. Genet. 4, 225–232.
Wimmer EA, 2005a. Insect transgenesis by site-specific recombination. Nature Meth. 2, 580–582.
Wimmer EA, 2005b. Eco-friendly insect management. Nat. Biotechnol. 23, 432–433.
Yuval B, Blay S, Kaspi R, 1996. Sperm transfer and storage in the Mediterranean fruit fly (Diptera: Tephritidae). Ann. Ent. Soc. Am. 89, 486–492.
Zhou Q, Haymer DS, 1998. Molecular structure of *yoyo*, a *gypsy*-like retrotransposon from the Mediterranean fruit fly, *Ceratitis capitata*. Genetica 101, 167–178.
Zwiebel LJ, Saccone G, Zacharopoulou A, Besansky NJ, Favia G, Collins FH, Louis C, Kafatos FC, 1995. The *white* gene of *Ceratitis capitata*: a phenotypic marker for germline transformation. Science 270, 2005–2008.