



Prospects for using genetic transformation for improved SIT and new biocontrol methods

Alfred M. Handler

Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, US Department of Agriculture, Gainesville, FL 32608, USA (Phone: +1-352-374-5793; Fax: +1-352-374-5794; E-mail: handler@nersp.nerdc.ufl.edu)

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Abstract

The genetic manipulation of non-drosophilid insect species is possible by the creation of recombinant DNA constructs that can be integrated into host genomes by several transposon-based vector systems. This technology will allow the development and testing of a variety of systems that can improve existing biological control methods, and the development of new highly efficient methods. For programs such as sterile insect technique (SIT), transgenic strains may include fluorescent protein marker genes for detection of released insects, and conditional gene expression systems that will result in male sterility and female lethality for genetic sexing. Conditional expression systems include the yeast GAL4 system and the bacterial Tet-off and Tet-on systems that can, respectively, negatively or positively regulate expression of genes for lethality or sterility depending on a dietary source of tetracycline. Importantly, strains for male sterility must also incorporate an effective system for genetic sexing, since typically, surviving females would remain fertile. Models for the use of these expression systems and associated genetic material come from studies in *Drosophila* and, while many of these systems should be transferable to other insects, continued research will be necessary in insects of interest to clone genes, optimize germ-line transformation, and perform vector stability studies and risk assessment for their release as transgenic strains.

Abbreviations: GFP – green fluorescent protein; *hAT* – *hobo*, *Ac*, *Tam3*; RFP – red fluorescent protein; rtTA – reverse tetracycline transcriptional activator; SIT – sterile insect technique; TRE – tetracycline response element; tTA – tetracycline transcriptional activator; UAS – upstream activating sequences; Yp – yolk protein.

Introduction

The ability to use recombinant DNA to molecularly engineer insects opens the door to a wide array of techniques to control pests and improve beneficial species and, in particular, create strains to improve biocontrol methods such as the sterile insect technique (SIT). Using strategies similar to those that employ classical genetic manipulations, the potential exists to create genetically transformed strains for genetic marking, male sterility and genetic sexing. The hope is that these strains will be simpler to create, have greater stability, and have improved viability and reproductive competitiveness.

Several factors have recently come together to make these ‘biotechnological’ strategies closer to reality. Foremost is the ability to genetically transform non-drosophilid insect species, most notably a wide variety of dipterans. Second are recent advances in the development and testing of several conditionally regulated gene expression systems in *Drosophila melanogaster* that, most likely, can be utilized in other insects. Tight conditional regulation is essential if we wish to have healthy viable strains for mass-rearing, that can be manipulated to kill female offspring and sterilize the remaining males, while maintaining male sexually activity and competitiveness.

This paper will provide an overview of the systems currently available for germ-line transformation in insects, genetic-marking systems available to select transgenic insects and identify them in field release programs, and some of the transcriptional activation systems that might be employed for genetic sexing and male sterilization. A general benefit of these types of approaches is that the fast-paced acquisition of genetic information should allow new and improved strategies to be continuously available with relatively rapid implementation. A drawback of these approaches is that they rely on the release of transgenic insects that will most certainly be met with social and scientific concern, making risk assessment and safety issues a high priority.

Insect gene-transfer

The genetic transformation of a wide variety of insects using several systems has been reviewed extensively in recent years (Ashburner, Hoy & Peloquin, 1998; Handler & James, 2000; Atkinson & O'Brochta, 2001; Handler, 2001) and thus only a brief overview will be presented here. The transformation systems available include germ-line transformation that results in the stable heritable integration of a transgene, as well as systems that allow the extrachromosomal transient expression of a genetic system, usually mediated by a viral or bacterial system. For the applied use of gene expression systems in released insects, germ-line transformation typically mediated by a transposable-element-based system is currently the method of choice. At present there are four transposon vector systems available for use in a wide variety of insects, that include *Hermes* from *Musca domestica* (Warren, Atkinson & O'Brochta, 1994), *mariner* from *Drosophila mauritiana* (Haymer & Marsh, 1986; Medhora, MacPeck & Hartl, 1988), *Minos* from *D. hydei* (Franz & Savakis, 1991), and *piggyBac* from *Trichoplusia ni* (Fraser, Smith & Summers, 1983). Curiously, the first transposon vectors studied for non-drosophilid transformation, *P* and *hobo*, had been discovered in *D. melanogaster* and were already in routine use in that species. Yet both of these elements were found to have no, or very limited function outside of *Drosophila*, while the other systems cited function effectively in both drosophilids and non-drosophilids (Handler, Gomez & O'Brochta, 1993; O'Brochta & Atkinson, 1996).

Regardless of their relative function, all of the transposon vectors are used in a similar fashion that includes a binary system of non-autonomous vector and helper transposase plasmids. The vector includes the inverted terminal repeat sequences and subterminal sequences needed for mobility, that surround a selectable marker gene and other sequences of interest. The transposase gene within the vector is either deleted or made defective and vector transposition depends upon a helper plasmid that contains the transposase gene but not the terminal sequences necessary for integration. Thus when transiently expressed in the germ-line, the helper transposase can catalyze integration of the vector but is lost in subsequent cell divisions allowing the integrated vector to remain stable.

Mariner and Minos

Of the transposons used as vectors in non-drosophilids, *mariner* was the first to be discovered, but its ability to transform *Drosophila* was limited (Lidholm, Lohe & Hartl, 1993), and it was several years before it was successfully tested in other species. Transformation was first achieved in the mosquito *Aedes aegypti* using the *kynurenine-hydroxylase*-white marker (Coates et al., 1998), and was later used to transform *M. domestica* (Yoshiyama, Honda & Kimura, 2000). The *Minos* element was the first transposon to be used for germ-line transformation of a non-drosophilid, that being the Mediterranean fruit fly, *C. capitata* using a *white*⁺ cDNA eye color marking system in the *white eye* host strain (Loukeris et al., 1995). *Minos* has subsequently been used to transform the mosquito *Anopheles stephensi* using a green fluorescent protein (GFP) marker (Catteruccia et al., 2000).

Hermes

The most widely used transposon vectors in non-drosophilid insects are *Hermes* and *piggyBac*, but their more extensive use and testing have also revealed information relating to their function and presence that could influence how they are used for applied purposes. The existence of *Hermes*, and other members of the *hobo*, *Ac*, *Tam3* (*hAT*) family, was inferred from the cross-mobilization of *hobo* in species where *hobo* did not exist (Atkinson, Warren & O'Brochta, 1993; see O'Brochta & Atkinson, 1996). *Hermes* and other *hAT* elements were then discovered by amplification of genomic elements using common amino acid sequences in *hobo* (from *Drosophila*) and *Ac*

(from maize) as priming sites. A complete *Hermes* element was first tested for function in *D. melanogaster* by germ-line transformation (O'Brochta et al., 1995), and its function in other species was tested by transient transposition tests (Sarkar et al., 1997). Subsequently, *Hermes* was used successfully to transform *A. aegypti* (Jasinskiene et al., 1998), *Stomoxys calcitrans* (O'Brochta, Atkinson & Lehane, 2000), *Tribolium castaneum* (Berghammer, Klingler & Wimmer, 1999), *C. capitata* (Michel et al., 2001) and *Culex quinquefasciatus* (Allen et al., 2001). While most of these transformations resulted from *Hermes*-mediated cut-and-paste transpositions, a curious finding was that the integrations in *A. aegypti* were not precise transposon-mediated events, but included rearranged integration of the entire vector plasmid by some type of recombination event. These unusual integrations, however, were still dependent upon the presence of *Hermes* transposase, and it has been theorized that an interaction between the injected *Hermes* and endogenous *hAT* elements resulted in replicative recombination events (Jasinskiene, Coates & James, 1999). Presuming the genes of interest within the vector are not disrupted by the recombination event, transformation by recombination is not necessarily a drawback, especially if it enhances transgene stability (and indeed, 'recombination' events are typically used for transformation in plant and vertebrate animal systems). Recombinant integrations are problematic if remobilization of the primary integration is desired for studies such as transposon-tagging and enhancer-trapping, and in applied use where the goal is to have autonomous functional vectors driven into a population (see Handler, 2001).

A more daunting consideration for the use of *Hermes* is related to how its existence was first theorized (Atkinson, Warren & O'Brochta, 1993), and this is the recent finding that *Hermes* and *hobo* can indeed cross-mobilize one another based on excision assays (Sundararajan, Atkinson & O'Brochta, 1999). Thus the possibility exists that *Hermes* integrations in species harboring functional *hAT* elements will not remain stable. Low level instability may not be problematic for small population experimental studies, especially using efficient visible markers for the transgene. However, even rare instability will be problematic for large mass reared populations in terms of maintaining strain integrity as well as program efficiency. The problem of potential vector instability is most apparent for *Hermes*, however, all the vectors in use are widely functional, and have the same

potential for instability and inter-species movement. Addressing this potential will be one of the primary needs for the effective and safe use of transgenic insects in release programs.

piggyBac

Similar to *mariner*, the *piggyBac* element was discovered long before its potential for non-drosophilid transformation became apparent. Notably, it was discovered by virtue of its ability to transpose from its host genome within a cabbage looper moth cell line, into an infecting baculovirus (Fraser, Smith & Summers, 1983; Cary et al., 1989). Later molecular analysis of the element (then called IFP2) and its functional characterization by transient mobility assays indicated that it could potentially be used as a vector for germ-line transformation in several orders of insects (see Fraser, 2000). Similar to *Minos*, it was first tested in medfly using the *white* marker system, but unlike the other vector systems that used heat shock regulated helpers, the *piggyBac* transformation used an unmodified transposase gene indicating that the *piggyBac* vector had autonomous function in different insect orders (Handler et al., 1998). Germ-line transformation with *piggyBac* was then achieved in other dipterans including *D. melanogaster* (Handler & Harrell, 1999), *Bactrocera dorsalis* (Handler & McCombs, 2000), *Anastrepha suspensa* (Handler & Harrell, 2001a), *M. domestica* (Hediger et al., 2001), *A. aegypti* (Lobo et al., 2002), and *Anopheles albimanus* (Perera, Harrell & Handler, 2002); two lepidopteran species, *Bombyx mori* (Tamura et al., 2000) and *Pectinophora gossypiella* (Peloquin et al., 2000); and a coleopteran, *T. castaneum* (Berghammer, Klingler & Wimmer, 1999). Unlike the other transposon vectors, *piggyBac* is not an apparent member of a widespread family of elements, though other elements share its specificity for integration into the tetranucleotide site, TTAA. Limited searches for *piggyBac* did not provide evidence for its existence beyond the cabbage looper moth, and thus it was somewhat surprising to find multiple elements nearly identical to *piggyBac* present in the genome of the oriental fruit fly, *B. dorsalis* (Handler & McCombs, 2000). Initial hybridization and PCR studies indicated that 10–20 elements existed in the genome of wild type and mutant strains, though none have been proven to be functional. Hybridization studies indicate that *piggyBac* also exists in another lepidopteran, *Spodoptera frugiperda*, though

these elements are probably highly truncated forms (A.M. Handler, unpublished). Thus, the existence of *piggyBac* in both closely and distantly related species suggests that it has recently traversed orders by horizontal transmission and probably exists in other species as well. This movement had to be facilitated by functional *piggyBac* elements or cross-mobilizing systems, and thus the considerations for *Hermes* stability must be extended to *piggyBac* as well.

Transgenic strains for biological control

Transgenic strains may be created to improve existing biocontrol programs such as SIT, or potentially allow new highly efficient control strategies. Most simply, marker genes can be integrated into strains allowing the unambiguous identification of released insects in traps (Handler & Harrell, 2001a). Other transgene constructs may enhance genetic sexing by the sex-specific production of a lethal gene product, or male sterilization by specifically destroying reproductive tissue. The high probability that these types of strains can be successfully created and manipulated is supported by the genetic manipulation of a variety of organisms, most notably *D. melanogaster*. The large number of transgenic strains created for this species not only provides a roadmap for methodologies and feasibility, it also provides an enormous archive of cloned material available for immediate application, either by direct use or use as probes to discover homologous genes in target species.

The basis for achieving both male sterility and female lethality for SIT is the development of vector constructs containing genes that result in cell death or disruption of cell division. Male sterility should result from the specific expression of such genes in sperm-producing cells of the testis, while female lethality should occur when specifically expressed in vital tissues of females but not males. Novel strategies for biocontrol have also been proposed whereby released insects and their offspring die or where only their offspring die or are sterile. There are a variety of mutant and normal genes affecting cell viability that can be used in these strategies, including mutant lethal genes affecting vital processes, normal genes involved in programmed cell death (White et al., 1994; White, Tahaoglu & Steller, 1996), and genes that express toxic molecules such as the diphtheria and ricin toxins (Kalb, DiBenedetto & Wolfner, 1993). A critical component to the use of these genes, however, is the ability

to regulate their expression in terms of developmental, tissue, and sex-specificity so that breeding populations can be maintained.

Genetic markers

The marking of released flies has generally been achieved by dusting pupae with a fluorescent powder that is transferred to the adult upon emergence (see Hagler & Jackson, 2001). This marking depends on powder caught in the ptilinum during emergence, which allows the most consistent retention of the powder. Typical problems with this method are that flies often groom themselves that may eliminate the powder, and the powder may be transferred to non-released flies, especially during mating. This can result in ambiguity when scoring insects caught in traps. There are also health concerns related to the effects of the powder on workers in mass-rearing facilities. Genetic markers that result in a new phenotype can eliminate these problems, but the marker should result from a dominant-acting gene and must be visible in adults. It is also important that the marker be apparent in insects caught in traps, which might be dead for several weeks before being examined. A fortuitous realization was that fluorescent protein markers developed for selecting transgenic insects could also be used for their identification after release. Currently, these markers include the jellyfish GFP (Prasher et al., 1992; Chalfie et al., 1994) and variants of this gene that result in enhanced green intensities and other colors (e.g., blue, cyan, yellow), and the coral, *Discosoma striata*, red fluorescent protein (DsRed or RFP) (Matz et al., 1999).

Most experimentation has been done on the GFPs, which have been linked to a variety of promoter systems. These include the polyubiquitin promoter that is expressed in all tissues throughout development (Handler & Harrell, 1999, 2001a, b), actin promoters expressed in all tissues (Peloquin et al., 2000; Pinkerton et al., 2000; Tamura et al., 2000), and the artificial 3xP3 promoter expressed from eye rhodopsin cells (Berghammer, Klingler & Wimmer, 1999; Horn, Jaunich & Wimmer, 2000). Ideally, gene expression should be from a variety of tissues since some body parts may be lost in trapped flies (e.g., heads, legs, wings, antennae), and expression should be stable and intense enough to be detected in dead flies. The polyubiquitin and actin promoters may be best suited in this regard, and tests with polyubiquitin-regulated EGFP in Caribbean fruit flies indicate that fluorescence from

the thoracic flight muscle can be detected in flies for as long as 4 weeks after death (A.M. Handler, unpublished). An advantage for all of the fluorescent protein marker systems is that the genes are completely exotic to insects, and thus sensitive molecular and biochemical methods can be used to make definitive identification if the visible phenotype is ambiguous. Another advantage of fluorescent protein markers is the recent development of an automated sorter of fluorescent insect embryos that can be adapted for any of the various proteins (Furlong, Profitt & Scott, 2001). This was developed for sorting *Drosophila* eggs carrying a marked chromosome, but could also be used for tephritids and possibly adapted for other insects. Such a sorting system could be used for sexing and could be a powerful method to rapidly identify transformants.

Transcriptional activation systems

Genetic systems that cause death or sterility must be tightly regulated so that breeding populations can survive and reproduce. This is generally achieved by conditional regulation where expression of the gene of interest is regulated by manipulation of temperature or chemical treatment, or by interbreeding two independent strains. Model systems for biocontrol have already been tested in *Drosophila* using temperature-sensitive lethal alleles and by creating female lethals and steriles by tetracycline-dependent transcriptional repression. Another transcriptional activation system under consideration is the GAL4 system in which the GAL4 regulatory protein promotes expression of lethal genes linked to a GAL4-specific enhancer sequence.

The tetracycline-resistance operon gene expression system. The tetracycline-resistance operon from the *E. coli* Tn10 transposon has been developed into positive and negative gene expression regulatory systems, resulting in either the promotion or inhibition of gene expression in the presence of tetracycline (or derivatives such as doxycycline) (Gossen & Bujard, 1992; Gossen et al., 1995) (see Figure 1). The components of this system include a transcriptional activator (tTA) that in its 'wild type' form is inhibited by tetracycline from binding to the *tet* operator sequence (*tetO*; or *tet* response element, TRE). Thus tetracycline represses expression of genes linked to the TRE (known as 'Tet-off'), and functionality for this system in *Drosophila* has been demonstrated (Bello, Resendez-Perez & Gehring, 1998). A mutated form of

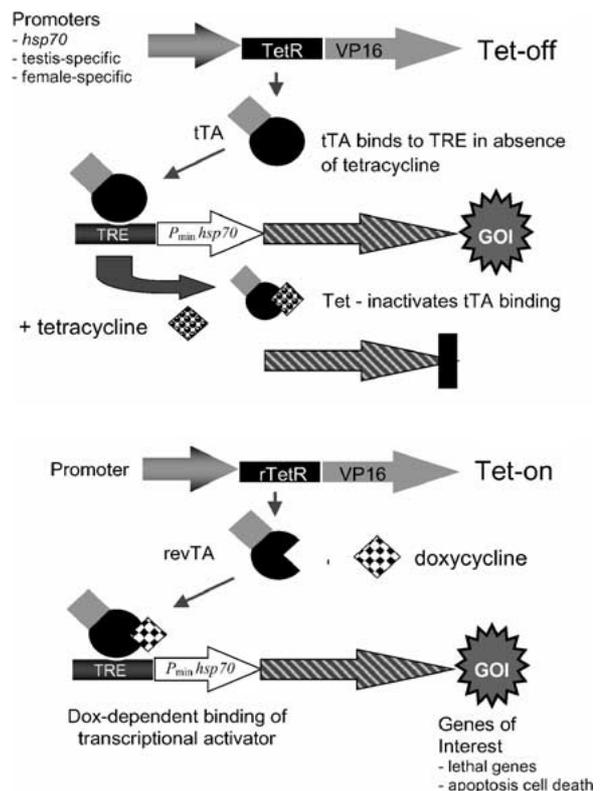


Figure 1. Diagram of the Tet-off and Tet-on conditional gene expression systems whereby expression of genes linked to the TRE are repressed by the presence of tetracycline or induced by the presence of doxycycline. See text for further explanation.

the tTA known as the *reverse* transcriptional activator (revTA or rTA), acts in a converse fashion in that rTA requires the presence of a tetracycline derivative, doxycycline (dox), to bind to the TRE (Kistner et al., 1996). In this 'Tet-on' system, gene expression is dependent on the presence of doxycycline, which has also been demonstrated in *Drosophila* (Bieschke, Wheeler & Tower, 1998). Thus, the expression of a TRE-linked gene of interest can be positively or negatively regulated by antibiotic depending on the type of transcriptional activator present (either tTA or rTA) and by how its production is regulated. In this way a cell death gene linked to the TRE would be produced in the absence of tetracycline with tTA, and in the presence of doxycycline with rTA.

Use of the Tet-off system for biocontrol has been reported in two studies using *Drosophila* as a model system to achieve female-specific lethality. Heinrich and Scott (2000) used the yolk protein 1 (Yp1) promoter to drive female-specific tTA, and linked the TRE enhancer to the cell death gene, *head involution defective* (*hid*) (Grether et al., 1995). In the

presence of tetracycline *hid* expression was repressed and both sexes survived, but in the absence of the antibiotic, nearly all females died during pupation or early adulthood in some strains, presumably due to *hid* expression in the female adult fat body and ovarian follicular epithelium. Strain differences in lethality were attributed to varying expression of the transgenes due to position effects, and it was also found that nutrition affected Yp1 promoter function. This scheme could certainly be optimized for efficient female-specific lethality, though its use for genetic sexing in SIT still may be impractical. Young adults are released for SIT, and females might not be dead at the time of release or easily distinguished from surviving males, and costs would still be incurred for larval rearing, sterilization, shipping and release, diminishing the advantages of this system.

Thomas et al. (2000) also showed in *Drosophila* that the Tet-off system could be used for biocontrol in a model system called 'release of insects carrying a dominant lethal' (RIDL), whereby female-specific lethality is also regulated by the presence of tetracycline. Similar to the system described above, it can be used as a genetic sexing system to enhance SIT, but was described primarily as a system to suppress populations directly (the Heinrich and Scott method also has this potential, but was not described or tested as such). Strains maintained on tetracycline could be released whereupon their progeny would die in the absence of antibiotic in the field. For this system the tTA was placed under promoter regulation of the heat shock gene, Hsp26, which is constitutively expressed in all tissues, and the yolk polypeptide 3 (Yp3) gene which, like Yp1, specifies expression in the female adult fat body and ovarian follicular epithelium (Tamura, Kunert & Postlethwait, 1985). Two genes were linked to the TRE; a dominant and ubiquitous cell lethal involved in signal transduction, Ras64B^{V12} (Fortini, Simon & Rubin, 1992), and a mutant allele of the *msh-2* dosage compensation gene, *msh-2*^{NOPU}, whose mis-expression causes female-specific lethality (Kelley et al., 1995). Appropriate transgenic strains were created and intermated, with progeny maintained on media containing tetracycline thereby repressing expression of the lethal genes. When removed from tetracycline, male progeny were apparently unaffected while none of female progeny survived from strains containing Yp3-tTA/TRE-Ras64B^{V12}, Hsp26-tTA/TRE-*msh-2*^{NOPU}, or Yp3-tTA/TRE-*msh-2*^{NOPU}.

As a method for genetic sexing, use of a Yp3-tTA/TRE-Ras64B^{V12} strain would have the same

drawbacks as use of a Yp1-tTA/TRE-*hid* strain, since Yp1 and Yp3 share the same regulatory specificities and female death would be expected in late pupae and young adults. However, *msh-2*^{NOPU} should function in early development and an Hsp26-tTA/TRE-*msh-2*^{NOPU} strain could allow efficient genetic sexing. An important caveat is that *msh-2* activity is probably regulated differently in other insects (in medfly in particular; see below), and it remains to be determined if this gene or its cognates will be useful for female-specific lethality.

In terms of population suppression, release of males carrying a Tet-repressed dominant female-lethal system would be expected to result in non-viability of their female offspring in the wild in the absence of tetracycline. It was argued that the efficacy of this strategy could be enhanced by having homozygous transgenes, integrations on multiple chromosomes and increasing release numbers, resulting in greater effectiveness than SIT. This comparison was made by a mathematical model, and such comparisons are difficult especially when extrapolating to other species in the wild. What is notable is that full suppression of a population using RIDL is not expected before the third generation in the best scenario, and thus, it would not be useful by itself as an immediate suppression strategy.

The practical use of the Tet-off system would have mass-reared parental strains raised on diet containing tetracycline to repress expression of sterility or lethality genes, with expression of these genes expected in emerging adults or earlier stages of progeny taken off diet. Use of the Tet-on system for practical application has not been described yet, but this system would allow a more selective expression of genes of interest by exposing insects to diet at specific times when gene expression is required. Conceivably last instar larvae could be treated in a drug-containing solution which could be repeatedly re-used. The original experimentation with the Tet systems in *Drosophila* indicates that the Tet-off system is more stringent (Bello, Resendez-Perez & Gehring, 1998), with some 'leakiness' described for Tet-on (Bieschke, Wheeler & Tower, 1998). Use of any of these systems will require optimization in terms of tight conditional expression, requiring a survey of independent integrations to control for position effects, and possibly multiple integrations to boost expression.

The yeast Gal4/UAS binary gene expression system. Another method for regulating gene expression that is functional in a variety of plant, animal, and insect

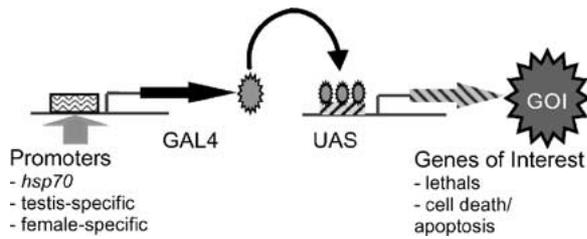


Figure 2. Diagram of the GAL4/UAS system for conditional gene expression whereby genes linked to the UAS are regulated by the GAL4 transcriptional regulatory protein. See text for further explanation.

cells is the Gal4/UAS transcriptional activation system from yeast (see Figure 2). Similar to the *tet* operon, GAL4 is a transcriptional activator that binds to a specific enhancer sequence resulting in transcription of coding sequences linked to that enhancer. This system has been optimized by mutations in the Gal4 gene and by mutations and reiterated sequences in the enhancer, known as the upstream activating sequences (UAS), to provide high affinity binding sites for the GAL4 protein (Brand & Perrimon, 1993). The expression of genes linked to the UAS enhancer is thus regulated by the expression of GAL4, which may be specified in turn by a variety of tissue-specific, sex-specific, or conditional promoters (Brand, Manoukian & Perrimon, 1994). To control gene expression the Gal4 and UAS components are separated in different strains, and only upon interbreeding can Gal4 driven by a promoter of interest, activate expression of a structural gene of interest linked to the UAS enhancer. Thus sex- or tissue-specific lethality can be achieved by having a transgenic strain with the appropriate UAS-linked gene mated to another transgenic strain with the Gal4 regulated by a sex- or tissue-specific promoter. A benefit of this approach is that no other external treatment is required, though a major drawback is the necessity to mate only one sex from each parental strain (i.e., Gal4 males crossed to UAS females) requiring a secondary system of sexing. Nevertheless, several hundred-fold fewer parental insects would have to be sexed relative to the number of offspring released.

Alternatives to SIT

Strategies for using transgenic insects for biocontrol have centered on improving existing methods such as SIT, but the true future potential for transgenic insects will be the development of new and novel strategies for

biocontrol that are highly efficient, and have minimal handling and rearing costs. Foremost among these will be conditional lethal strains, in which released flies and their offspring die in response to a change in their permissive rearing conditions. An example of this is the Tet-off-regulated lethality described above, where successive generations of female offspring die in the absence of tetracycline. Another novel approach, known as autocidal biological control (ABC), utilizes a dominant-acting temperature-sensitive lethal gene (Fryxell & Miller, 1995). This is a cold-sensitive lethal allele of the *Notch* gene, *Notch*^{60g11}, that results in lethality of both heterozygous and homozygous individuals at temperatures of 18°C and below. Thus insects may be reared and released at 24°C and above, but they and their offspring would be expected to die as ambient temperatures decrease. An important caveat to all the systems described is that they have only been modeled, and in some cases, tested in *Drosophila*. Although many of the genes tested in *Drosophila* are expected to be conserved, these genes may not function, or function optimally in other species requiring their cloning from the insects of interest.

Genetic reagents for biocontrol

The promoters and structural genes used to achieve genetic sexing or male sterility in gene expression systems should be the same for any particular species. For the examples used in *Drosophila*, the tissue- and sex-specific promoters as well as genes causing cell death should function similarly and be interchangeable among the Tet-on, Tet-off, or Gal4 regulated systems. In addition to genes tested in these model systems, other genes exist that can be substituted or used in combination to achieve the same effect.

Cell lethality. Two cell death genes that interact with *hid* in *Drosophila* to elicit programmed cell death, or apoptosis, during development and during pathological processes are *reaper* (*rpr*) and *grim* (Abrams et al., 1993; White, Tahaoglu & Steller, 1996; Wing et al., 1998). Their activity results in the elimination of cells for the sculpting of tissues during development, and probably plays a major role in the histolysis of larval tissue during metamorphosis (Jiang, Baehrecke & Thummel, 1997). These genes, and several others, share a conserved sequence known as the 'death domain', and encode proteins that initiate a cascade of interactions that include activation of cysteine proteases known as ICE/CED-3-like

proteases (Bump et al., 1995; Pronk et al., 1996; Kondo, Yokokura & Nagata, 1997). Notably, death occurs in organisms when these genes do not function, or if they function in inappropriate tissues or times in development. Of particular relevance has been the demonstration of tissue ablation resulting from inappropriate *rpr* and *hid* expression (Zhou et al., 1997; Nassif et al., 1998; Wing et al., 1998). Cell death genes act autonomously in specific tissues, and many share significant structural and functional homologies (Evans et al., 1997; Kondo, Yokokura & Nagata, 1997; Vucic, Seshagiri & Miller, 1997). Optimism for *rpr* and *hid* having a lethal effect when ectopically expressed in transgenic insects is based on *Drosophila rpr* inducing cell death when transiently expressed in a lepidopteran (*S. frugiperda*) cell line (Vucic, Seshagiri & Miller, 1997). *Reaper* was also shown to interact in an apoptosis cascade in the vertebrate, *Xenopus* (Evans et al., 1997). Thus it is highly likely that the *Drosophila* cell death genes will function similarly in other dipterans, though it should be a straightforward process to isolate analogous genomic clones from other insect species. Similarly, a variety of other genes have been isolated from *Drosophila* having vital functions, such as in signal transduction and other roles in programmed cell death (Bonini & Fortini, 1999). Many of them may be used to induce cell lethality, and testing will be required to discover those that are most efficient, and whether cognates must be cloned from the insect of interest.

A direct approach towards achieving cell-specific lethality is the use of toxin genes such as diphtheria and ricin, which should be widely effective. For both toxins, the subunit A moiety of the protein is toxic, and may be expressed in the absence of the subunit B moiety that allows trans-membrane movement. Without the subunit B, the toxic effect is cell-specific, prohibiting damage to adjacent tissue or to predatory organisms. Temperature-sensitive alleles also exist for these toxins allowing the potential for added levels of conditional expression. For diphtheria toxin, heat-sensitive alleles allow toxicity only at temperatures below 20°C (Bellen et al., 1992), while for ricin, converse-acting cold-sensitive alleles are only toxic at 20°C or above (Moffat et al., 1992), and these alleles could be used in strategies similar to the ABC technique. The advantage of toxin genes is that they are highly active in heterologous systems, allowing their immediate use in practically all insect species. In contrast, cell death and other lethal genes from *Drosophila* have a higher likelihood of having a diminished or lack

of function in other species, and certainly for those that are distantly related.

Sex-specificity. For the objectives of male sterility or female lethality, genes expressed specifically in males or females are necessary to utilize their sex-specific regulatory promoters. For some genes, especially those involved in sex determination or dosage compensation, sex-specific intron-splicing mechanisms and sex-specific gene product function may also be manipulated for these purposes.

Testis-specific expression: To achieve male sterility it is necessary to have a regulatory system expressed specifically in the male reproductive system, preferably in a tissue involved in spermatogenesis. If expression of a lethal gene is desired, then there must be very high tissue-specificity for expression so that male viability is not compromised and the function of associated tissues involved in male reproduction are unaffected. Two genes known to be testis-specific are $\beta 2$ -*tubulin* and *Sdic*.

Tubulins are structural proteins within all cells that are integral to microtubule formation necessary for chromosome movement. Several tubulin isotypes are specific to particular tissues and, in some cases, developmental periods. In *Drosophila*, a $\beta 1$ -*tubulin* is expressed in all tissues, including larval germ-line and somatic tissue early in testis stem cell differentiation, but at the beginning of spermatocyte formation in the third larval instar, a switch occurs to the $\beta 2$ isotype specifically in the germ-line (Buttgereit & Renkawitz-Pohl, 1993; Fackenthal, Turner & Raff, 1993; Hoyle et al., 1995). Mutations of $\beta 2$ -*tubulin*, which are indicative of their tissue-specificity, produce sterile males with immotile sperm resulting from a disorganization of spermatid components and abnormal axonemal microtubular structure in developing spermatozoa (Castrillon et al., 1993; Fackenthal et al., 1995). Such testes exhibit significant meiotic defects due to a failure of microtubule function, including a failure to form the meiotic spindle, improper or absent chromosome movement, and failure to undergo cytokinesis (Kemphues et al., 1982). Viability of such mutant males is not affected and thus $\beta 2$ -*tubulin*-regulated cell death gene expression should only affect spermatogenesis in the male testis. An interesting feature of microtubules is that they are sensitive to both the dosage and type of tubulin isotypes available and in spermatocytes, overexpression of $\beta 2$ -*tubulin* or mis-expression of a non- $\beta 2$ -*tubulin* isotype results in sterility. Notably, ectopic expression of a $\beta 2$ -*tubulin*

cognate cDNA from *Heliothis virescens*, regulated by the *Drosophila* $\beta 2$ -*tubulin* promoter, also caused sterility in *Drosophila* (Raff et al., 1997). Thus, the potential exists to elicit male sterility by manipulating tubulin expression in the testes, using the $\beta 2$ -*tubulin* regulatory system.

Another gene having expression totally limited to the male testes in *Drosophila* is *Sdic*, which was discovered as a unique gene encoding a novel axonemal dynein intermediate chain expressed specifically in spermatocytes (Nurminsky et al., 1998). At present it is unknown if this gene exists in other insects or organisms, or if *Drosophila Sdic* will be functional in other insects. Conservation between the *Sdic* and $\beta 2$ -*tubulin* promoters suggests that function will be maintained, and possibly its promoter can be interchanged with that of $\beta 2$ -*tubulin*.

Female-specific expression: Following the male paradigm, female-specific expression can also be achieved by using the regulatory promoter region from genes specifically expressed in females. Alternatively, it can also take advantage of manipulating sex determination gene expression, or the sex-specific RNA splicing mechanisms that are used to direct sex determination gene product function (Baker, 1989; Handler, 1992). For genetic sexing that relies on female lethality, female-specific promoters are needed that function in all tissues or in tissues that are vital for survival. As in males, most sex-specific promoters in females function in reproductive tissue that may be used to cause sterility, but not organismal lethality. The only genes known to be female-specifically expressed in a vital tissue are the yolk protein (Yp) genes that were discussed previously. They are expressed in the female adult fat body of almost all insects, where a lethal effect would be expected to occur, as well as in the ovarian follicular epithelium of some Diptera. Since Yp expression is usually limited to late pupae or adults, this is problematic for genetic sexing systems where female lethality is desired in early development to avoid rearing costs. It has also been discovered in some dipteran candidates for SIT, including the Caribbean fruit fly (Handler, 1997) and the stablefly (Chen et al., 1987), that Yp production is totally limited to the ovaries, and thus Yp-regulated lethal genes would probably only cause sterility. Undoubtedly this ovarian-specificity for Yp promoter function will extend to other species as well.

A more promising strategy to achieve female lethality in early development is to utilize female-specific mRNA intron splicing systems used for

female-specific sex determination gene expression. These systems were first discovered in introns within the *Drosophila transformer (tra)* and *doublesex (dsx)* sex determination genes (Burtis & Baker, 1989; Sosnowski et al., 1994), which function throughout female development beginning in early embryogenesis (Baker, 1989). The female-specific function of these genes depends upon the utilization of an alternative 3' splice site that is only recognized in females (Ryner & Baker, 1991; Sosnowski et al., 1994; Heinrichs & Baker, 1995). The sequence in between this splice site and an upstream 3' site recognized in males, encodes a translational stop signal. Thus, this stop site remains in male transcripts, resulting in premature polypeptide termination. In females, the stop signal is spliced out with the intron allowing complete translation resulting in a functional protein product. By fusing sequence 3' to the intron in-frame with another coding sequence, we expect the complete translation of that coding region only to occur in females. Thus, if such a fusion is made with a gene causing cell lethality, it should only be functional in females resulting in death. One potential problem for use of the *tra* gene splicing mechanism is that it is leaky, resulting in the occasional use of the male splice site in females (Sosnowski, Belote & McKeown, 1989), which would reduce the level of cell lethal gene expression. Importantly, a system analogous to *dsx* having a sex-specific splicing mechanism has been isolated and described for the Queensland fruit fly, *B. tryoni* (Shearman & Frommer, 1998). It is likely that either the female-splicing systems in *B. tryoni* will be functional in the medfly and other tephritid species, or that the *dsx* system can also be isolated from these species.

Another novel method of achieving female-specific lethality is to manipulate genes involved in dosage compensation, as was described earlier for *msl-2^{NOPU}* (Thomas et al., 2000). In *Drosophila*, dosage compensation occurs in males by the male-specific lethal complex (MSL) acting to hyperactivate expression of hemizygous X-linked genes (Franke & Baker, 2000). The MSL genes are normally suppressed in females by *Sex-lethal (Sxl)*, but if this suppression fails, females will die due to over-expression of their X-linked genes, while males will remain unaffected. The *msl-2^{NOPU}* allele remains active in females due to deletion of its *Sxl* binding sites. A caveat for use of this system is that dosage compensation mechanisms vary widely among animals (e.g., mammals use X-inactivation in females), and in some insects the females are heterogametic. Thus the genes involved

in dosage compensation or the manner in which they function are quite different among insects (Marin, Siegal & Baker, 2000). Indeed, while *Sxl* acts female-specifically to suppress MSL function in *D. melanogaster*, it is expressed in both males and females of medfly and several other dipterans (Saccone et al., 1998 and this volume). Thus, MSL-type genes may not have a function in male dosage compensation in these species, but if they do, their suppression in females must occur by a different mechanism.

Sex determination genes: Theoretically, an ideal strain for SIT could result from the manipulation of the sex determining genes themselves (see Handler, 1992; Chapter by Shearman). This is based upon a temperature-sensitive mutation of *transformer-2* (*tra-2^{ts}*) in *Drosophila* (Belote & Baker, 1982; Belote et al., 1985). For *tra-2^{ts}* homozygotes reared at permissive temperatures of about 25°C and below, male and female development is normal, but at the restrictive temperature of 29°C, XX chromosomal females develop as sterile males, while XY males are also sterile. Thus a breeding population may be maintained at 25°C, with all progeny developing as sterile males when shifted to 29°C. While such a scheme does not necessarily require transgenic strains, gene replacement strategies with genes mutated *in vitro* may be required to most easily duplicate them in other species. Presently, suboptimal fitness of existing mutant strains in *Drosophila* would not make them ideal candidates for large scale rearing, though strain creation by transformation would avoid secondary genetic defects inherent in mutagenesis selections. Such a scheme, however, does provide a model for the future development of the most highly efficient strains for SIT, and the creation of novel strategies for biological control.

Summary

The ability to genetically manipulate insects presents many possibilities for creating transgenic strains for improved biological control. The first of these strains will improve existing methods such as SIT, by allowing genetic marking, male sterility and genetic sexing. Creation and use of genetic markers based on fluorescent protein genes should be straightforward, and the current use of these markers for transformant selection already provides some strains for this purpose. Strains for male sterility using transcriptional activation systems also have a high probability for success, though testis-specific expression will probably require

promoter systems cloned from species of interest. A highly important consideration for the development of male sterile strains is that they will only be practical when used in conjunction with an efficient method for genetic sexing. If a male-specific mechanism is used for sterility, eliminating the need for irradiating flies for release, then fertile females must be completely eliminated. For medfly, the *tsl*-based sexing system (Franz et al., 1996; Franz, this volume) could be used with a transgenic male sterile system, but for other species *tsl*-based strategies would likely take several years to develop, and may be impractical to even attempt. Transgenic strains for female-specific lethality should be possible for most species, but these are also prospective and will require further basic research into female-specific promoters, and mechanisms regulating sex determination and dosage compensation. The discovery that the *B. tryoni* and *D. melanogaster doublesex* genes are conserved gives optimism to the notion that female-specific splicing systems can be used to regulate lethality in many tephritid species.

While there is good reason to be optimistic that genetically altered strains can be created to improve SIT and biocontrol systems in general, there are caveats that are important to address. Much of this optimism is based on gene function and models studied in *Drosophila*, and this is especially so for genes involved in sex determination or dosage compensation. While many of these genes, like *dsx*, should have similar functional and regulatory properties in other species, many of them will vary to the extent that cognates will have to be cloned from the species of interest, and some, like *Sxl*, will be functionally distinct. Transformation studies will be essential to determining functional relatedness, and important to identifying related genes in insects of interest.

The second major caveat to consider is transgene and transgenic strain stability, which relates in large part to the regulation and mobility properties of the transformation vector system. Most of the transposon-based vectors in use are part of families where inter-species horizontal movement has occurred, and for at least one, cross-mobilization between different elements has been demonstrated. Thus, vectors may be introduced into species or strains where genomic instability can result in strain breakdown and/or vector movement into another host organism. This would dramatically affect program effectiveness, and have important implications for ecological risk assessment that may potentially limit the release of transgenic

insects. These uncertainties require continued research into the regulation and behavior of transposable elements, and especially in systems where they are not normally found. Other avenues of research include testing systems that can, most straightforwardly, render vectors immobile after their initial genomic integration.

In conclusion, the use of recombinant DNA technology with recent advances in insect transformation methods gives great hope for the development of new transgenic strains that will revolutionize our ability to control insect populations. The most efficient use of this technology, however, will require continued studies into vital genetic pathways involved in insect development, reproduction, sex determination, and dosage compensation. The safe and effective use of transgenic strains for this purpose will require continued studies into the regulation and host range of the transformation vectors used for their creation.

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