Improving the Ecological Safety of Transgenic Insects for Field Release: New Vectors for Stability and Genomic Targeting

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ABSTRACT Genetically transformed insect pests provide significant opportunities to create strains to improve the sterile insect technique (SIT) and new strategies based on conditional lethality. A major concern for programmes that rely on the release of transgenic insects is the stability of the transgene, and maintenance of consistent expression of genes of interest within the transgene. Transgene instability could influence the integrity of the transformant strain upon which the effectiveness of the biological control programme depends. Loss or intragenomic transgene movement could result in strain attributes important to the programme being lost or diminished, and the mass-release of such insects could significantly exacerbate the insect pest problem. Instability resulting in intragenomic movement may also be a prelude to intergenomic transgene movement between species resulting in ecological risks. This is a minor concern for short-term releases where transgenic insects should not survive in the environment beyond one or two generations, but transgene movement may occur into infectious agents during mass-rearing, and the potential for movement after release is a possibility for programmes using many millions of insects. Random genomic insertion is also problematic for transgenic strain development due to genomic position effects that influence transgene expression, and insertional mutations that negatively affect host fitness and viability. New types of vectors are described that allow post-integration immobilization by deleting terminal vector sequences required for transposition, and genomic targeting by a recombinase-mediated cassette exchange strategy.

KEY WORDS biological control, transposable elements, transgenic strains, sterile insect technique, conditional lethality, insect transformation, vector stabilization, vector targeting, recombinase-mediated cassette exchange

1. Introduction

In recent years the development of transgenic insect strains has advanced rapidly, with more than 20 species within four orders of insects being genetically transformed (Handler 2001). The ability to efficiently introduce recombinant DNA into insect host genomes provides significant opportunities to study the genetic basis of insect biology in a wide range of species, in ways previously limited to model insect systems such as *Drosophila*. Gene transfer also provides the opportunity to create transgenic strains that may be used directly to control the population size or behaviour of agriculturally and medically important insects. Transgenic strains may be created to improve existing biological control strategies, such as the sterile insect technique (SIT), or to provide the means for new strategies for biological control based on conditional lethality (Alphey 2002, Handler 2002a). For beneficial insects, their vigour and reproductive capacity may be enhanced, in addition to their ability to produce and process proteins. In some cases, such as vectors of dis-

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ease, instead of suppressing the population of pest insects, they may be transformed into inhospitable hosts for the parasites or pathogens that they normally transmit (James 2005).

The significant advances in basic and applied studies that genetically transformed insects may provide must, however, be viewed in light of several limitations that are inherent to the gene transfer vector systems used to integrate transgenes into the host genome (Handler 2004). All of the heritable germ-line transformations in insects have been achieved with vectors derived from a type of mobile DNA known as transposable elements, which include the elements *Hermes*, *mariner*, *Minos*, and *piggyBac*. While these elements provide advantages over other types of vectors and transformation strategies, a major consideration is their potential for remobilization which can compromise the stability of the transgenic host strain, and thus adversely affect programme effectiveness. While the transposase enzyme required for transposon movement is typically eliminated after integration, the undetected or unintended presence of the transposase or related enzymes within the host can result in vector remobilization. While generally not considered to be a problem for small-scale laboratory studies, the rearing and release of many millions of insects for biological control programmes increases the probability that such rare events may occur. Other caveats relate to the generally random nature of transposon integration into host genomes. Localized genomic effects on gene expression can result in variable transgene expression depending upon the integration site. Vector integrations into coding regions or important regulatory regions can result in mutations having deleterious effects on the transformed host's fitness and viability. Thus random integrations make comparative gene expression studies problematic, and greatly reduce the efficiency of creating optimal strains for applied use.

To address these limitations on transgenic insects, new vectors have been developed that can be stabilized subsequent to genomic integration, and for which defined integration target sites may be created within the genome. These, and similar strategies by other laboratories should provide a new generation of vectors that both increase the efficiency of transgenic strain development, and at the same time, increase the effectiveness of transgenic strains and their ecological safety.

2. Transgenic Insects for Biological Control

Genetically transformed insect strains have great potential for improving existing biological control programmes for pest species such as those integrating the SIT, or to develop new control strategies based on the conditional regulation of genes that encode lethal products (Handler 2002a). For beneficial insects, the potential exists to develop transgenic strains having enhanced immune systems, increased longevity and reproductive capacity, or heightened response to odorant cues elicited by prey insects.

Vectors of disease may also be eliminated by biological control methods, or potentially, allowed to exist but made refractory to the parasites and pathogens that they normally transmit so that they no longer threaten human or animal health (James 2005). Several of these strategies are discussed in more detail in this volume (Aksoy et al., Alphey et al., Bourtzis et al., this volume), but briefly, lethality induced by transgenic techniques can improve the SIT by allowing more efficient genetic sexing by causing lethality specifically in females, or for male sterilization by specifically eliminating tissues required for fertility (Handler 2002a). Alternatively, genes involved in sexual differentiation, or sex determination, may be manipulated so that the sexual phenotype is disrupted or reversed (Handler 1992, Pane et al. 2005). Most straightforwardly, transgenic strains marked with green fluorescent protein (GFP) or red fluorescent protein (DsRed), initially used to select transformants, may also be used to unambiguously identify insects in the field after release (Horn et al. 2002). This in itself

would be a major advance over using fluorescent powders for marking (Hagler and Jackson 2001). Novel strategies for biological control have also been proposed whereby the offspring of mass-released insects either die or are sterile (Heinrich and Scott 2000, Thomas et al. 2000, Horn and Wimmer 2003). A variety of mutant and normal genes affecting cell viability are potentially useful for these strategies, including genes involved in programmed cell death (White et al. 1994), genes encoding toxin subunits such as diphtheria (Kalb et al. 1993), or mutations that cause a normal gene product, such as *DTS-5* (Saville and Belote 1993) or *Notch*^{60g11} (Fryxell and Miller 1994), to become toxic at either high or low temperature, respectively.

A critical component of these strategies is the regulated expression of the lethal product, both to maintain breeding populations in facilities and to target the lethal phenotype to a particular tissue or stage in development. This can be achieved by making transgene activity conditional to a particular temperature range, chemical treatment, or by the interbreeding of specific genotypes. Such methods have already been tested in *Drosophila*, and include the use of temperature sensitive lethal alleles or the use of ectopic transcriptional regulators such as the *Gal4/UAS* system (Brand et al. 1994) or the *Tet-off/on* systems (Bello et al. 1998) that respond to dietary tetracycline.

3. Transposon Vectors and Insect Transformation

To better understand the limitations and risks, as well as the advantages, associated with genetically transformed insects, it is helpful to understand the methods and mechanisms used to create them. All of the heritable transformations of insect germ-lines have utilized Class II transposable elements as vectors that transpose by a DNA-mediated "cut-and-paste" process (Atkinson et al. 2001). These mobile genetic elements are typically one to three kilobases in length and have terminal sequences that are inverted repeats of one another. The terminal inverted repeats are usually 30 base pairs or less, but some are several hundred base pairs and some terminal regions also have subterminal inverted repeat sequences. The terminal inverted repeats and adjacent DNA are excised and reinserted together into a new DNA insertion site as part of the transposition process. In between the terminal inverted repeats is a gene for a transposase enzyme that binds to the terminal sequences to catalyse both the "cut-and-paste" processes. In this way, most transposons are self-contained autonomous elements that may require other host-encoded nuclear proteins. Importantly, while the terminal inverted repeats and transposase gene are usually linked as a *cis*-acting unit, the terminal inverted repeats and intervening DNA can be mobilized by an unlinked transposase gene acting in *trans*. This feature has allowed the development of defective non-autonomous vector plasmids that only include the terminal inverted repeats, marker genes, and genes of interest with its transposase gene either mutated or deleted. These vectors can then only be mobilized by a separate source of transposase helper, provided by a plasmid-encoded gene lacking terminal inverted repeats, or the transposase RNA or protein. When co-injected into preblastoderm embryos, the transposase catalyses integration of the vector, but does not integrate itself in the absence of terminal inverted repeats, and is eventually diluted with cell division. Once the transposase is lost, vector integrations into the germ-line chromosomes should remain stable.

All insect transformations to date have utilized transposon vectors, though the first two vectors originally discovered and used in *Drosophila melanogaster* Meigen, *P* (Rubin and Spradling 1982) and *hobo* (Blackman et al. 1989), have not been found to be effective in other species (Handler 2001). Other functional elements that have been used in non-drosophilids depended on the fortuitous discovery of new transposons, or the directed search for *hobo*-related elements in non-drosophilids. These include the *Minos* element discovered in *Drosophila hydei* Sturtevant (Franz and Savakis 1991), which is

closely related to *Tc* elements from nematodes, and the *Mos1 mariner* element from *Drosophila mauritiana* Tsacas & David (Medhora et al. 1988). Elements from the *hobo*, *Ac*, *Tam3* (*hAT*) family include *Hermes*, discovered in the house fly *Musca domestica* L. (Warren et al. 1994) and *Herves*, recently discovered in *Anopheles gambiae* Giles (Arensburger et al. 2005). *Hermes* is widely functional, but quite importantly, it has been shown to functionally interact with *hobo* (Sundararajan et al. 1999), providing some of the strongest experimental evidence to support the need for methods to stabilize transgene integrations.

The most widely used transposon vector to date is the *piggyBac* element discovered in a baculovirus passed through a cell line of the cabbage looper *Trichoplusia ni* (Hübner) (Fraser et al. 1983, Cary et al. 1989). A *piggyBac* vector was first used to transform several tephritid species, and use of a lepidopteran transposon in dipteran species portended the broad functionality of this element, which has been proven by its use in nearly 20 species within four orders of insects (Handler 2002b). Molecular analysis of *Bactrocera dorsalis* Hendel transformants, however, indicated the potential for cross-mobilization in this species since Southern hybridizations showed genomic sequences in the host strain that were closely related to *piggyBac* (Handler and McCombs 2000). This was confirmed by polymerase chain reaction (PCR) analysis, and further studies now show that *piggyBac* elements, having greater than 95% nucleotide identity, exist throughout the *B. dorsalis* complex and several other closely related species (G. Zimowska and A. Handler, unpublished).

The finding of a moth transposon in dipteran species suggested that *piggyBac* might also exist in other moths, and this has been confirmed by Southern hybridization and sequence analysis of elements isolated by PCR (G. Zimowska and A. Handler, unpublished). These species include *Helicoverpa zea* (Boddie), *Helicoverpa armigera* (Hübner), and *Spodoptera frugiperda* (J. E. Smith), in addition to new elements discovered in *T. ni*. This is a strong indication that *piggyBac* has been horizontally transmitted between distantly related species, and for this to occur, functional elements must exist in these species or associated organisms. As with *Hermes*, these findings raise the concern for potential remobilization and instability of transgenes vectored by the respective transposons. While the existing data raise most concern for *Hermes* and *piggyBac*, both the *Minos* (Avancini et al. 1996) and *mariner* (Robertson and MacCleod 1993) elements also exist in broad, potentially functionally compatible, families of elements, and unless proven otherwise the concerns for transposon vector stability must be extended to these, and possibly all future transposon vectors as well.

4. Methods to Stabilize Transposon Vectors

While genetically transformed insects present a wide array of possibilities to create strains with attributes that can greatly improve existing biological control methods and the development of new strategies for control, the effective use of such strains will depend on the reliable expression of the integrated genes of interest, as well as maintenance of strain fitness and viability under mass-rearing protocols. It is also critical that the transgene vector is stably integrated to maintain strain integrity and to prevent possible interspecies movement of the transgene into unintended hosts, which is a major concern for ecological safety. Current knowledge of known transposons, and especially those used for insect transformation, makes these concerns of primary importance.

The major contributing factor to vector instability is most likely the presence of the same transposon, or a functionally related system, in the host genome or in an associated infectious or symbiotic organism within the host. The former possibility can be tested by direct structural tests using DNA hybridization or PCR analyses to detect the same element or a related element with a high degree of sequence identity. If a related system is

functionally conserved but lacking sufficient structural identity for easy detection, as would be the case for *hobo* and *Hermes*, functional assays may be performed. Indeed, transposition and excision assays that show mobility in the host in the absence of an exogenous source of transposase (i.e. by injecting only donor and target plasmids for transposition, or indicator plasmid for excision) provide the most straightforward test for cross-mobilization (Atkinson et al. 1993). However, these assays are typically performed in cell lines and embryos, and are probably not sensitive enough to detect mobility catalysed by a non-host source of transposase, and especially from coexisting organisms that proliferate post-embryogenesis. Given these caveats, it is highly unlikely that the complete potential for transgene vector remobilization can be definitively and unambiguously assessed, leaving open the possibility, regardless of how minimal, that transgenic insertions will not remain stable. This creates a significant point of concern for the ecological risk assessment that will be required for a transgenic release certification. Indeed, addressing the issues of potential transgene instability and interspecies movement was a primary concern in response to the environmental assessment for the release of a transgenic pink bollworm *Pectinophora gossypiella* (Saunders), solicited by United States Department of Agriculture's Animal and Plant Health Inspection Service-Plant Protection and Quarantine (USDA-APHIS-PPQ). The inability to adequately address these issues had led us and others to consider development of a new generation of vectors that can be immobilized, with respect to transposase activity, after initial genomic integration has been achieved.

4.1. Vector Immobilization

Immobilization or stabilization of a transposon vector is most straightforwardly achieved by deleting or rearranging DNA within the vector that is required for transposition. This includes the terminal inverted repeats and possibly additional adjacent DNA. For most transposons this includes up to 100 base pairs of terminal sequence (though longer for elements such as *Minos* whose terminal inverted repeats themselves are 255 base pairs). Deletion or rearrangement of these sequences is most simply achieved by introducing short sequences that specifically recombine with one another in the presence of an appropriate enzyme. Two examples of these systems are the *FRT*/FLP recombinase system from the two micron plasmid of yeast (Andrews et al. 1985) and the bacteriophage Cre-*loxP* system (Siegal and Hartl 1996). A functional *FRT* recombination site consists of two 13 base pair inverted repeats separated by an eight base pair spacer that specifically recombine with one another in the presence of FLP recombinase. Depending upon their location and orientation, *FRT* recombination can result in chromosomal rearrangements or the targeting of a plasmid carrying an FRT to a genomic *FRT* site (Rong and Golic 2000). Recombination of *FRT*s in direct orientation results in the deletion of the intervening DNA, while *FRT*s in the opposite orientation results in inversions. It should thus be possible to position FRTs in vectors to create rearrangements within a vector, or between two independent vectors after their genomic insertion by injection of plasmid-encoded FLP recombinase (Handler 2004). While theoretically attractive, use of recombination systems is not simple. Placement of recombination sites within the vector may be difficult without negatively affecting its ability to integrate initially (due to disruption of the terminal sequence). Rearrangement between independent vectors is more plausible, but requires the vectors to be linked closely on the same chromosome with recombination sites in an indirect orientation, to avoid lethal deletions resulting from directly oriented sites. With recombination sites in indirect orientation, an *FRT* inversion between distantly integrated vectors has been achieved in *Drosophila* resulting in stabilization of both vectors since the inversion reconstitutes chimeric vectors, each having a terminal sequence of the other

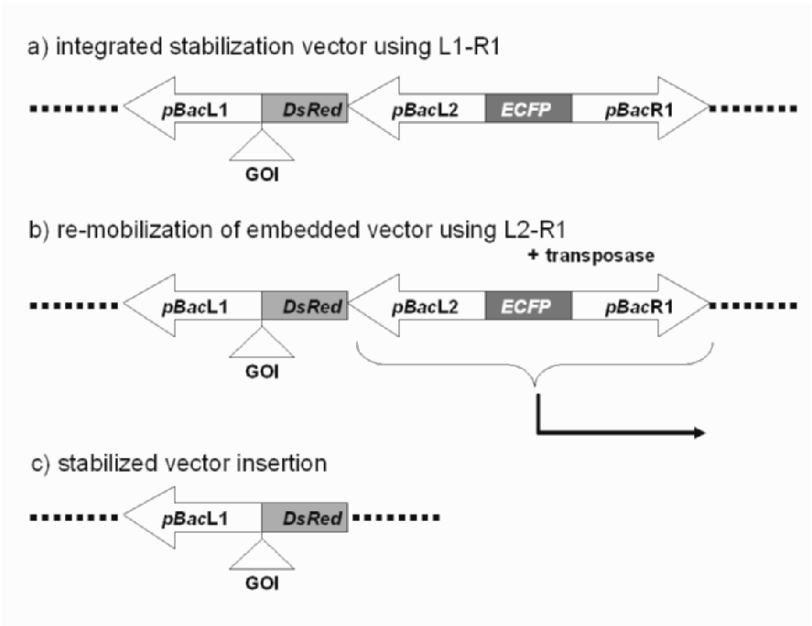

Figure 1. *Transgene stabilization by terminal sequence deletion of the pBac{L1-PUbDsRed1-L2-3xP3-ECFP-R1} vector. The diagram (not to scale) shows relative positions of the pBacL1, pBacL2 and pBacR1* piggyBac *terminal sequences, the PUbDsRed1 and 3xP3-ECFP markers, and an insertion site for genes of interest. Transposase is provided by either mating to a piggyBac jumpstarter strain, or by injection of a* piggyBac *helper plasmid. Integration of the entire stabilization vector is determined by the presence of both DsRed and ECFP markers, and terminal sequence deletion is determined by remobilization of the L2-3xP3-ECFP-R1 embedded vector resulting in loss of the ECFP phenotype. The genomic stabilized transgenes include the pBacL1 terminus, the DsRed marker, and any inserted gene of interest.*

(E. Wimmer, personal communication).

This approach has the added advantage of creating a balancer chromosome (within the inversion) in which normal recombination is suppressed in heterozygotes. This is a very encouraging result using an elegant approach to achieve vector stabilization, but its success in *Drosophila* will be difficult to repeat in other insects where inserting linked vectors, and mapping and determining vector orientation is much more difficult. This will be ameliorated to some extent in insects whose genome has been sequenced. In order to simplify and extend the use of vector stabilization to many species, another approach has been taken that results in terminal sequence deletion without the use of recombination sites.

4.2. Vector Stabilization by Terminal Sequence Deletion

To stabilize transposon vectors subsequent to genomic integration, a method to delete a terminal vector sequence required for mobility was first tested in *Drosophila* by introducing an internal tandem duplication of the other terminal sequence, with independent fluorescent protein markers placed between each set of termini (Handler et al. 2004) as shown in Fig. 1. Specifically, the *piggyBac* vector, pBac{L1-PUbDsRed1-L2-3xP3-ECFP-R1}, was created by placing a duplicated 5' terminal *piggyBac*

sequence (pBacL2) internal to the flanking 5' (pBacL1) and 3' (pBacR1) termini, with independent markers placed between each set of termini. Genes of interest to be stably integrated would be placed between the duplicated termini. Transformation with this vector can result in two types of integration: either the shorter embedded L2-3xP3-ECFP-R1 sequence may integrate by itself, or the entire L1-PUbDsRed1-L2-3xP3-ECFP-R1 vector may integrate.

In general, shorter vectors transpose more efficiently than longer vectors, and indeed, transformation with this vector resulted in seven lines with only the embedded L2-3xP3-ECFP-R1 vector, and one line with the entire L1-PUbDsRed1-L2-3xP3-ECFP-R1 vector. However, after mating the L1-PUbDsRed1-L2-3xP3-ECFP-R1 strain to a *piggyBac* transposase jumpstarter strain (having a chromosomal source of the functional transposase gene), the L2-3xP3-ECFP-R1 vector was remobilized resulting in progeny having only the L1-PUbDsRed1 transgene sequence genomically integrated. In the absence of the R1 3' *piggyBac* terminus, it was expected that the remaining genomically integrated sequence would remain stable with respect to remobilization by a source of transposase. This was tested by mating the stabilized line to the jumpstarter strain, which showed that no remobilization occurred (by loss of phenotype) in more than 7000 progeny assayed. This compared to about 5% remobilization rate in the original L1-PUbDsRed1-L2-3xP3-ECFP-R1 vector. This showed that the transgene was stabilized owing to the loss of the 3' *piggyBac* terminus. A similar stabilization vector has been integrated into the Caribbean fruit fly *Anastrepha suspensa* (Loew) and the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) with the embedded vectors remobilized by injection of transposase helper plasmid. The testing for stability in resulting progeny of these species is in progress.

5. Vector Targeting

A major difficulty in creating optimal transgenic strains for biological control is decreased fitness and viability due to vector integrations disrupting vital gene functions, and diminished or altered transgene expression due to genomic position effects. Both drawbacks can be minimized by having transgene integrations limited to defined genomic target sites known to be devoid of vital DNA and subject to minimal position effects. To target a plasmid donor vector to a specific genomic locus an FLP recombinase-mediated cassette exchange (RMCE) system (see Baer and Bode 2001) was modified for use in insects, and tested in *Drosophila* (Horn and Handler 2005). A recombinase-mediated cassette exchange system is based upon double recombination between small recombination sites (such as *FRT* or *loxP*) within a genomic target site, and a plasmid donor sequence as shown in Fig. 2.

A *linotte* homing sequence from *Drosophila* was added since such sequences placed within a plasmid vector are known to target the same endogenous genomic sequences, and it was reasoned that this might enhance recombination between the donor plasmid and the genomic target site. Target site strains were created by transformation with a *piggyBac* vector (pBac{3xP3-FRT-ECFP-linotte-FRT3}) having two heterospecific *FRT* recombination sites (*FRT* and *FRT3*) surrounding an enhanced cyan fluorescent protein (ECFP) marker coding region and the linotte homing sequence. Transformant lines from embryos having an integrated target vector were then injected with a donor vector plasmid (pSL-FRT-EYFP-linotte-FRT3) having corresponding *FRT/FRT3* sites surrounding an enhanced yellow fluorescent protein (EYFP) marker coding region and *linotte* sequences. Recombination between the target and donor *FRT/FRT3* sites was mediated by co-injection of an FLP recombinase helper plasmid (pKhsp82-FLP).

Targeting of the genomic acceptor site by recombination with the donor plasmid was determined in the progeny of the injected embryos, with recombinants identified at a frequency of about 23% by screening for conversion of the enhanced cyan fluorescent protein to the enhanced yellow fluorescent pro-

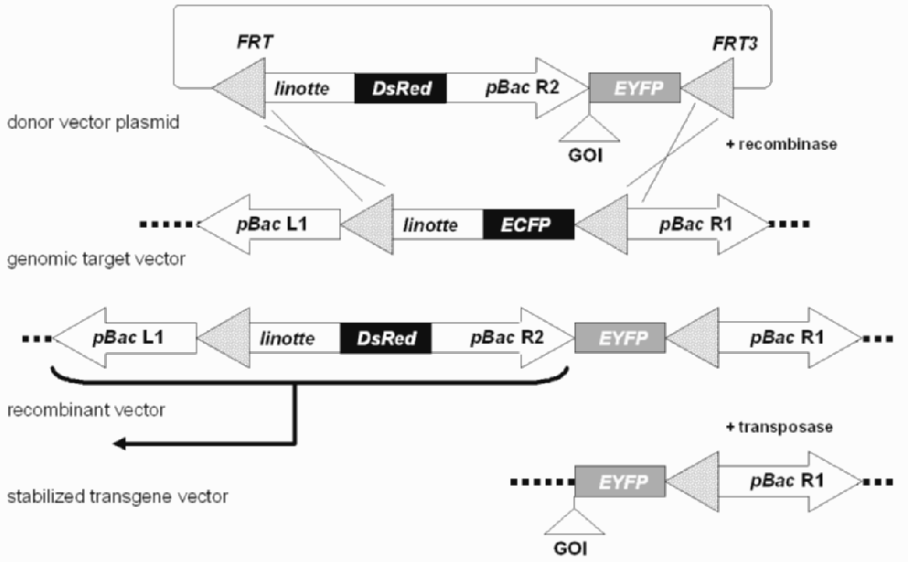

Figure 2. *Genomic targeting by recombinase-mediated cassette exchange (RMCE) and subsequent target site vector stabilization by terminal sequence deletion. The genomic target vector, pBac{3xP3-FRT-ECFP-linotte-FRT3}, is first integrated into a host genome by transposase-mediated germ-line transformation. It is then targeted by recombinase-mediated cassette exchange by co-injection of the donor vector plasmid {pSL-FRT-EYFP-linotte-FRT3}, and the FLP recombinase helper plasmid, pKhsp82-FLP, into target strain embryos (see text for details of plasmid constructions). Recombinants are identified by the exchange of the enhanced cyan fluorescent protein (ECFP) marker in the target strain for the DsRed and enhanced yellow fluorescent protein (EYFP) markers introduced by* FRT/FRT3 *double-recombination with the donor vector. The genomic target site is subsequently stabilized by transposase-mediated remobilization of the pBacL1 terminus from the target vector and a pBacR2 terminus from the donor vector (Fig. 1), which is determined by loss of the DsRed phenotype. The genomic stabilized transgenes from this strategy include the pBacR1 terminus, the enhanced yellow fluorescent protein marker, and any genes of interest (GOI) inserted into the donor vector.*

tein eye fluorescence marker phenotype. However, in addition to cassette exchange products from double reciprocal *FRT* and *FRT3* crossovers, integration products from single *FRT* crossovers were also identified, but these could be discriminated by separable fluorescent markers (e.g. a DsRed marker placed outside the *FRT*s in the donor plasmid; not shown in Fig. 2). To stabilize targeted insertions, a new recombinase-mediated cassette exchange donor vector had a *piggyBac* 5'-terminus incorporated to allow post-integration deletion of the *piggyBac* 3'-terminus as described above. New transgene vectors such as these, that allow genomic targeting and post-integration stabilization, should significantly improve the efficient creation and safety of insects intended for field release.

6. Conclusions

The use of transgenic insect strains to improve the biological control of insect pests has enormous potential for success, but the development and release of such transgenic strains must be approached with a very high level of caution. While the actual risk of transgene remobilization may be very small, the large

number of insects used for field release programmes, the inability to retrieve these insects once released, and the known potential for remobilization of defective vectors certainly heightens the real and perceived concerns for transgenic release. Methods for vector stabilization after initial genomic integration by recombinase-mediated cassette exchange as described here, or by intervector recombination being developed by other laboratories, should eliminate the major cause of vector instability resulting from the unintended presence of transposase. In addition, vectors that allow genomic targeting should not only increase the efficiency of creating transgenic strains for biological control, but should also enhance the ability to compare and monitor transgene expression and stability between strains. This should improve the evaluation of transgenic strains for ecological safety as part of risk assessment protocols, and strain effectiveness during development and implementation. Thus, new vectors that allow both genomic targeting and subsequent stabilization should provide a significant advance in the development of transgenic insect strains for biological control.

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