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Developing the Arsenal Against Pest and Vector Dipterans: Inputs of Transgenic and Paratransgenic Biotechnologies

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Abstract

Insects are the most numerous of all animals and are found in almost every inhabitable place on earth. The order Diptera (true flies) contains many members that are notorious agricultural pests, nuisance or vectors of diseases. The list is long: mosquitoes, tsetse flies, screw worms, fruit flies, sand flies, blow flies, house flies, gall and biting midges, black flies, leaf miners, horse flies, and so on. Efforts to combat some of these pests and vectors have resulted in control measures such as the chemical, physical, and cultural control methods. These methods, though largely beneficial, have disadvantages and limitations, which sometimes seem to outweigh the problems initially sought to be controlled. The chemical method, for example, is not environment-friendly since it negatively affects many nontarget organisms and disrupts ecosystem balance. Development of insecticide resistance by pests/vectors is another concern. Molecular biotechnology has introduced vast arrays of novel ways to tackle pests and disease vectors, as well as improve the potency of existing control methods. This chapter looks at transgenic and paratransgenic biotechnologies and how they have been applied so far to develop and expand the arsenal against dipteran pests and disease vectors. Further, we discuss the advantages, disadvantages, and limitations of these technologies.

Keywords: insects, dipterans, crop pests, disease vectors, transgenesis, paratransgenesis

1. Introduction

Insects are highly abundant and are the most numerous classes of all described living animals. They account for about half or more of all living animals and are found in almost every inhabitable place on earth [1, 2]. Their success and abundance could be attributed

to their ability to adapt and colonize diverse habitats. Among insects, the order Diptera is one of the largest orders with an estimated 120 families and 250,000 described species [1]. They are generally regarded as the two-winged insects or true flies [2, 3]. The main characteristics for members of this order include larvae that lack legs (apodous maggots), pupae enclosed in a thick larval cuticle (puparium), and adults that possess a pair of membranous forewings, vestigial hindwing modified into halteres, as well as a tubular sucking or sponging mouthparts [2]. Dipterans are longtime foes and arguably considered the insect arch-enemy of man. This stems from the fact that many members of this order constitute pests of cultivated crops, are major causes of annoyance or are highly notorious as vectors of human diseases either in their larval or adult stages. Examples of pest, annoyance causing and vector dipterans are given in **Table 1** [4–8].

Efforts by man in the fight against dipteran pest and vector insects have resulted in the generation of an arsenal with several weapons. These range from chemical method which involves the use of insecticides to cultural methods such as sanitation, physical interference or destruction of breeding sites, and cropping methods. However, many of these methods have major disadvantages and (or) limitations that sometime seem to outweigh their benefits. For example, the chemical method is very widely used, but has the disadvantages of environmental consequences such as pollution, health challenges on man and livestock, killing of nontarget insect species, as well as the challenge of the targeted insect species developing resistance to the insecticides applied [9, 10]. Most cultural methods applied against dipteran pest or vector control are labor-intensive and can only be most suitably applied on a small scale.

Biologically-based approaches are generally friendlier to the environment, more sustainable and cost-effective than many other methods used for dipteran control. Here, control methods such as the use of natural enemies like predators and parasitoids are environment-friendly with varying levels of success, but the major limitation is the fact that it is unpredictable as chances are usually low on finding a suitable parasitoid or predator that can survive the weather and conditions wherever the pest or vector dipteran is and continue to effectively eat or parasitize the host [10]. The time it takes to find a good parasitoid may be so long that farmers or entomologists concerned may opt for other control methods, in addition to the fact that the process of actual control by a parasitoid or predator itself is slow. The biological method of using pathogens (microbial or biopesticides) has been quite promising, but recently there has been concerns of insect resistance as is the case with *Bacillus thuringiensis*, and also the disadvantage that the applied pathogen may infect other nontarget insects, livestock, or man himself. Major limitations of biopesticides are usually that one may need to find an efficient way to get the pathogens to their host and that the pathogens may be negatively affected by environmental conditions such as weather.

Another biologically-oriented and environment-friendly method for controlling dipterans is the use of pheromones or suitable attractants. However, the scale of its application and area that it covers is also limited, while the potency of the attractants does reduce gradually with time or could easily be influenced by environmental factors such as rainfall or masked by other chemicals within the vicinity.

Family	Genus/species involved	Problematic or damage-causing stage; problem caused
Agromyzidae (leaf miners)	<i>Phytomyza angelicastris</i> ; <i>Melani agromyza</i>	Larva; damage to leaves of crops
Anthomyiidae	<i>Antherigona</i> spp; <i>Delia radicum</i>	Larva; damage to stems of crops like cauliflower and sorghum causing disease like "dead heart"
Calliphoridae (blow flies)	<i>Callitroga</i> spp.; <i>Cordylobia anthropophaga</i> ; <i>Lucilia</i> spp; <i>Chrysomya bezziana</i>	Larva; myiasis or flesh infesting damage to man and livestock
Cecidomyiidae (gall midges)	<i>Contarinia sorghicola</i>	Larva; damage to leaves of crops such as rice, pear, sorghum, etc.
Ceratopogonidae (biting midges)		Adult; blood sucker from man and livestock
Chloropidae (chloropid flies)		Larva; damage to leaves of crops like rice and cereals
Culicidae (mosquitoes)	<i>Anopheles</i> spp.; <i>Aedes</i> spp.; <i>Culex</i> spp.; <i>Mansonia</i> spp.; <i>Psorophora</i> spp.; <i>Stegomyia</i> spp	Adult; blood sucker from man and livestock transmitting parasites that cause various diseases like malaria, dengue fever, West Nile fever, yellow fever, encephalitis, O'nyong nyong fever, Bancroftian filariasis, chikungunya, Igbo-Ora, Zika, etc.; nuisance, major cause of disturbance and annoyance to man at night
Drosophilidae	<i>Drosophila suzukii</i>	Larva; damage to fruits
Glossinidae (tsetse flies)	<i>Glossina</i> spp.	Adult; blood sucker from man and livestock transmitting the causative agent of Trypanosomiasis (sleeping sickness)
Muscidae (house flies)	<i>Musca domestica</i>	Adult; transmits microorganisms that cause cholera and amoebic dysentery; nuisance, major cause of annoyance to man during the day
Oestridae (warble or bot flies)	<i>Oestrus ovis</i> ; <i>Gasterophilus</i> spp.; <i>Hypoderma bovis</i> ; <i>Dermatobia hominis</i>	Larva; myiasis or flesh infesting damage to man and livestock
Psychodidae (sand flies and moth flies)	<i>Phlebotomus</i> spp.	Adult; blood sucker from man transmitting the parasite causing disease leishmaniasis
Sarcophagidae (flesh flies or screw worms)	<i>Cochliomyia hominivorax</i> ; <i>Sarcophaga</i> spp; <i>Wohlfahrtia</i> spp.	Larva; myiasis or flesh infesting damage to man and livestock
Simuliidae (black flies)	<i>Simulium</i> spp.	Adult; blood sucker from man transmitting the causative agent of the disease onchocerciasis (river blindness)
Tabanidae (horse flies)	<i>Tabanus</i> spp.; <i>Haematopota</i> spp.; <i>Chrysops</i> spp.	Adult; blood sucker from man and livestock transmitting the causative agent of diseases like trypanosomiasis (sleeping sickness) and loasis
Tephritidae (fruit flies)	<i>Anastrepha</i> spp.; <i>Bactrocera</i> spp.; <i>Ceratitis</i> spp.; <i>Dacus</i> spp.; <i>Rhagoletis</i> spp.; <i>Tephritis</i> spp.	Larva; serious damage to fruits and vegetables

Table 1. Dipteran crop pests, nuisance or vectors of diseases [4–8].

Genetic methods such as the sterile insect technique (SIT) majorly use radiation to sterilize male insects and thus reduce the fertility of females that mate with them [11, 12]. This method works well with sexually reproducing insects and has so far has great success among many dipterans [13]. Its major problem is the fact that the gamma radiation used for sterilization also reduces the fitness of the males and makes them less competitive than the wild males.

Obviously, no present method of pest control is devoid of disadvantages or limitations. As such, an integration of different control methods that are compatible is the recent paradigm. Integrated pest management (IPM) has offered a way to augment control methods to achieve a more efficient and sustainable management of pests and vectors.

The new millennium has witnessed advanced progress in genetic biotechnology which in turn has greatly influenced insect control. Biotechnology approaches have been used and are continually being pursued as a means to develop novel ways or improve some of the methods used to fight pest and vector dipterans. For example, new strains of reproductively sterile insects or strains exhibiting other desired traits could be engineered to control a population or designed to fit into control methods like SIT, entomopathogens or biopesticides that are adversely affected by weather conditions where a pest or vector is located or have environmental concerns regarding nontarget insects could be encapsulated in materials that will release the pathogens only in a desired condition, while nonharmful microorganisms could be engineered to deliver therapeutic or antiparasitic molecules to pathogens in their environment. Many of these new biotechnology approaches could also be used as a part of IPM programs which is suitable for other methods. In this chapter, we focus on how transgenic and paratransgenic biotechnologies have been applied to expand the array of weapons in man's arsenal against dipteran crop pests and vectors of diseases.

2. Transgenic biotechnology

Transgenesis aims at the transformation of an organism by altering its genetic composition and the final outcome is the generation of a transgenic or a genetically modified organism (GMO). Basically, desired genes or genes-of-interest from a different organism(s) are inserted into the genome of a wild type organism majorly with the aid of "jumping genes" called transposable elements or transposons and the transgenic organism generated carry these desired genes (transgenes), while exhibiting characters or traits encoded by the transgenes as well (**Figure 1**). For insects, germline transformation is sought and microinjections are performed to achieve it, allowing the genome modification to be passed on from generation to generation [14]. To enable detection of successfully transformed organisms, fluorescent proteins such as the green fluorescent protein (GFP), the red fluorescent protein (RFP), and fluorescent proteins of other colors are used as markers [15–18]. Consequently, GM dipterans harboring a transgene that incorporates a fluorescent protein gene cassette as marker would express the fluorescent protein and can be visualized under a fluorescent microscope (**Figure 2**).

Figure 1. Schematic representation of transgenesis: desired gene-of-interest is cloned into a vector to generate a plasmid or transgene construct which is then microinjected into embryos. Adults developing from the injected embryos are outcrossed to non-injected ones and their progeny are screened. Progeny that are stably germline transformed express traits encoded by the genes in the plasmid construct injected, for example a red fluorescent protein, and as such are distinguished from untransformed ones.

2.1. Transgenic strategies against agricultural crop pest dipterans

2.1.1. *Drosophilids*

The family Drosophilidae consists of many members including the well-known model fly *Drosophila melanogaster*, but only the spotted wing drosophila, *Drosophila suzukii*, is considered a major pest of cultivated crops [19]. However, *D. melanogaster* has been immensely beneficial in genetic studies and many proofs-of-principle of transgenic strategies against dipteran population control, or even for other insect orders, have been developed in this model insect.

A proof-of-principle transgene-based, embryo-specific lethality system for insect control was developed by Horn and Wimmer [20]. The system used embryo stage-specific promoters such as serendipity alpha (*sryα*) to regulate the expression of a *hidAla5* lethal effector placed under the control of a tetracycline-response element [20]. Such a strain would effectively achieve reproductive sterility in insect populations because the offspring die during the embryo stage and could replace radiation sterilization of insects as is the case for conventional SIT.

Two proofs-of-principle for transgenic sex-specific lethality systems for insect population control were developed: (i) using female-specific enhancers of yolk protein 1 (*yp1*) gene to drive expression of a *hid* effector under control of tetracycline-responsive element [21]. (ii) using a female-specific yolk protein and fat-body enhancer *Yp3* to regulate the expression of a *Ras64BV12* effector under control of tetracycline-responsive element, as well as using a

Figure 2. A transgenic strain of the Mediterranean fruit fly *Ceratitis capitata*: (A) visualized under cold light, (B) visualized under fluorescent light, the same transgenic fly shows a pattern of expression of green fluorescent protein GFP in its thorax and legs.

Hsp26 promoter to regulate expression of a dose-compensation gene, mutant male-specific lethal 2 (*msl-2NOPU*), under the control of a tetracycline-response element [22]. These kinds of systems limit lethality or death of offspring to only female individuals and could be used for efficient sex separation of dipterans prior to field release during area-wide dipteran pest control programs such as SIT.

Besides these afore-mentioned transgenic lethality systems which were all based on the tetracycline-repressible binary expression system (**Figure 3**) [23], a gene-driven system capable of driving population replacement was also developed in *Drosophila* [24]. Basically, a gene-driven system such as a maternal-effect dominant embryonic arrest (Medea) system use a combination of two genes that encode for a toxin and its antidote, respectively, to create a condition whereby a heterozygote female would express only the maternal toxin in half of her oocytes without the antidote resulting in death of those offspring. The Medea strain which was developed by Chen et al. in *Drosophila* used microRNA-mediated silencing of a maternally expressed embryonic development gene, *my88*, as its toxin and early zygotic expression of a rescuing transgene as the antidote. A more complex Medea system employing additional mechanisms such as targeting signaling pathways like the Notch pathway has since been also demonstrated in *D. melanogaster* [25].

Figure 3. Diagrams showing different versions of tetracycline-repressible expression system: (A) binary component tet-off system, (B) single component tet-off system [23, 29]. In the absence of tetracycline, tetracycline-repressible transactivator (tTA) is produced and goes on to bind to the tetracycline-response element (TRE) to activate expression of a downstream gene. Both systems are turned off in the presence of tetracycline which binds to the tTA and stops expression of a downstream gene. The binary system needs an effector for lethality, while the single component uses tTA which is toxic at high concentration.

For the crop pest Drosophilid, the spotted wing drosophila *D. suzukii*, germline transformation has recently been performed and transgenic strains for control of this strain may soon be generated [26].

2.1.2. Tephritid fruit flies

Tephritids are very important pests of fruits and vegetables and majority of transgenic strategies for crop pests have been developed against members of this group. Lethality systems that their proofs-of-principle have earlier been developed in *Drosophila* have also been successfully transferred to many Tephritids. Among these are the conditional embryonic lethality strains transferred from *D. melanogaster* to both the Mediterranean fruit fly, *Ceratitidis capitata* and the Caribbean fruit fly, *Anastrepha suspensa*, and using the tetracycline-regulated binary expression system, embryonic promoters/enhancers and proapoptotic hid effector [27, 28]. In addition, the lethality strains not previously established in *Drosophila* was developed for *C. capitata* using a simplification of the tetracycline-regulated binary expression system to a single expression component that relies on auto feedback-driven overexpression of a version of the tetracycline-repressible transactivator (tTA) for its lethality (Figure 3) [29].

For sex separation of Tephritids, transgenic sexing strains were developed for different fruit fly genera: (i) an RNA interference (RNAi) system developed for *C. capitata* based on knockdown

of transcripts of the sex determination gene transformer (*tra*) [30], (ii) lethality systems relying on a simplified single component tetracycline expression system and developed for *C. capitata* and the olive fruit fly, *Bactrocera oleae* [31, 32], (iii) lethality systems relying on a tetracycline-regulated binary expression system [23], embryonic promoters/enhancers and proapoptotic *hid* effector, and developed for *C. capitata*, *A. suspensa*, and the Mexican fruit fly, *Anastrepha ludens* [33–35]. Unlike the two proof-of-principle transgenic sexing systems based on lethality earlier developed in *Drosophila*, all the afore-mentioned transgenic sexing systems based on lethality in Tephritids employed the sex-specifically spliced intron of the gene transformer (*tra*) to confer lethality only to the female individuals. However, only those systems employing the tetracycline-regulated binary expression system and embryonic promoters or enhancers achieved female-specific lethality in the embryo stage [33–35]. Another type of transgenic sex-specific lethality system has recently been developed for the Oriental fruit fly, *Bactrocera dorsalis* [36]. This system combined the mechanism of alternative splicing of the double sex (*dsx*) gene and the toxicity of expressed ricin to ensure female-specific lethality and kill off the female progeny in *B. dorsalis* [36].

Since area-wide dipteran pest control strategies like SIT involve release of sterile males, a way to monitor the released males is also as important as the sterilization and sex separation of the males. Scolari et al. developed a transgenic strain that would facilitate such monitoring in *C. capitata* by using the promoter of a sperm-specifically expressed gene β 2-tubulin (β 2t) to regulate the expression of RFP and GFP to only male testis. Males of this sperm-marked fly strain were shown to still have brightly glowing fluorescent testis for several months after they had died [37]. As such, the released males could easily be monitored if caught in traps or found dead in the field in the case they were used in any SIT control program.

2.2. Transgenic strategies against dipterans of medical and veterinary importance

2.2.1. Mosquitoes

The battle against any other dipteran insects has perhaps never been as intense as it is for mosquitoes due to the wide range of diseases they vector and transmit. Almost every kind of approach that is imaginable is under development or has been developed in the effort to win the battle against mosquitoes. Ever since the first germline transformation of an *Anopheles* mosquito [38], several transgenic strategies have been constructed including gene drive systems, lethality, flightless, sperm-monitoring, as well as spermless systems, and mosquito strains that have been impaired in their ability to transmit a parasite.

Among the gene-driven systems include a maternally-regulated transposition system in the yellow fever mosquito, *Aedes aegypti*, which utilized regulatory elements of a maternal gene *Nanos* to control events in mosquito embryos [39]. A synthetic gene drive system developed in the human malaria mosquito, *Anopheles gambiae*, exploited I-SceI which is a selfish genetic element known as a homing endonuclease gene (HEG) to drive rapid invasion of mosquito population genomes by the engineered gene of interest [40]. The recently developed clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas), better known as the CRISPR-Cas9 system that enables flexible genome editing in both prokaryotic and eukaryotic cells [41–43] using a guide-RNA to direct the nuclease to its

target has also been exploited to develop a gene drive system with high transmission rate to progeny of up to 99.6% in *An. gambiae* [44].

Strains exhibiting dominant lethality or major incapacitation in the form of a flightless phenotype have also been generated. A transgenic strain based on the expression of dominant lethality in *Ae. aegypti* was constructed using similar components (a single expression component that relies on auto feedback-driven overexpression of a version of tTA) as was used for the Mediterranean fruit fly *C. capitata* [29, 45]. Fu et al. also generated a flightless strain of *Ae. aegypti* for dengue fever control by using its Actin4 (Act4) gene promoter in the single component tet-off expression system (**Figure 3**) to regulate the expression of tTA [46]. Act4 is female-specifically expressed in the indirect flight muscles [47], and as such, the tTA-mediated lethality regulated by its promoter is obtained predominantly in the female indirect flight muscles rendering them flightless and providing a way to genetically separate the sexes or enable possible male-only mosquito release in an SIT program [46].

For transgenic sexing and sperm monitoring, Catteruccia et al. established a strain that exhibited fluorescent sperms in the Asian malaria mosquito *Anopheles stephensi*, employing the promoter/enhancer elements of the $\beta 2$ tubulin gene to control and ensure expression of enhanced green fluorescent protein (EGFP) in male testis [48]. This has been followed by further sperm manipulation whereby to study possible roles of sperms in regulation of postmating female responses in the major malaria mosquito *An. gambiae*, a spermless strain was engineered by RNAi-mediated silencing of a developmental gene required for early germ cell differentiation, zero population growth (zpg) [49]. Mosquito control programs may benefit from the interesting report that female mosquitoes mated to the spermless males become refractory to further mating [49]. Moreover, such a spermless mosquito strain also possesses reproductive sterility and could also find application in SIT programs for mosquito population control. A very recent sex distorting system developed in *An. gambiae* employs the CRISPR/Cas9 endonuclease to shred the X chromosome and lead to male bias in progeny without significantly reducing the adult's fertility [50].

Another strategy that has also been pursued is to transgenically impair the ability of mosquitoes to transmit malaria Plasmodium parasites. To this end, transgenic Anophelines were developed that were unable to vector Plasmodium parasites as they express an antiparasitic peptide, the salivary gland and midgut peptide 1 (SM1) in their midgut epithelia under regulation by a carboxypeptidase promoter [51]. In the wake of insecticide and drug resistance by both vector and parasite, respectively, this approach offered an avenue to curtail transmission while not removing the vector and could easily be spread to wild mosquito population using some of the gene drive systems developed. Several other researchers have followed this strategy and developed transgenic mosquitoes that cannot transmit their parasites. Bee venom phospholipases, synthetic antimalaria proteins like vida3, single chain antibodies (scFv) targeting malaria parasites, as well as an antimicrobial peptide cecropin A have been used as effectors and mosquitoes engineered to express them lack the ability to effectively transmit parasites [52–56]. RNAi-based resistance to dengue virus has also been engineered in *Ae. aegypti* mosquitoes by using inverted-repeat RNA (IR-RNA) from the premembrane protein coding region of the DENV-2 RNA genome whose expression

was regulated by a carboxypeptidase promoter to suppressed viral replication in the midgut [57].

2.2.2. Blow flies

Veterinary pests such as Blow flies that inflict enormous damage to sheep and other livestock have also received attention lately. Transgenic sexing strains that allow male-only production for control of the Australian sheep blow fly *Lucilia cuprina* were developed using both the single and binary component tetracycline-repressible expression system. An initial single component female-specific lethality system showing lethality in pupa used a heat shock promoter Hsp70 and the transformer (tra) intron from *Cochliomyia hominivorax* to limit lethality to only females [58, 59]. A later strain which used the binary component of the tetracycline-repressible expression system and showed lethality in embryos utilized promoters of cellularization genes to drive expression of tTA and the transformer (tra) intron from *C. hominivorax* placed inside a hidAla2 effector gene to confer the lethality to only female individuals [60].

2.2.3. Screwworms

Though the very successful strategy of SIT had originally been developed against the New World Screwworm *C. hominivorax* [11], this has not translated into success in development of transgenic strains of this insect. Transformation of *C. hominivorax* is much more challenging than other dipterans and efforts have resulted in few transgenic strains that allow genetic marking with fluorescent proteins for management and control of screwworms [61, 62]. It is expected that similar transgenic sterile male strains, sexing strains, sperm marked, and organ-ismal lethal strains will be developed for screwworms in the near future as had already been done for other dipterans [62].

3. Paratransgenic biotechnology

Similar to transgenesis, paratransgenesis also involves the genetic transformation of organisms. However, paratransgenesis targets to achieve the genetic transformation or transgenesis of the symbionts that live inside an insect instead of the insect itself and cause the symbionts to express or secrete substances that act against parasites and pathogens that are transmitted by the insect (**Figure 4**). Consequently, paratransgenesis is suitably applied against disease vectors. Originally developed against the triatomine bug vector of Chagas disease, *Rhodnius prolixus* using its symbiont *Rhodococcus rhodnii* and the antimicrobial peptide cecropin A as an effector [63], this strategy has been adopted for many dipterans that vector diseases of humans and livestock.

3.1. Paratransgenic strategies against medical and veterinary important dipterans

3.1.1. Mosquitoes

Since mosquitoes transmit several disease-causing pathogens, many paratransgenic studies have been conducted on it. Inhibition of vectorial competence in mosquitoes via bacterial

Figure 4. Schematic representation of paratransgenesis: transgenes encoding anti-parasitic molecules are cloned into a plasmid which is used to transform suitable symbionts of a vector insect. When the transformed symbiont is introduced into the gut of the vector, they colonize the gut whereas the anti-parasitic molecules they produce act against the parasites and clear them off.

symbiont paratransgenesis were demonstrated in the malaria mosquito *An. stephensi* using genetically modified strains of the popular gram negative bacteria *Escherichia coli* to express scFvs that block development of the parasite *Plasmodium berghei* [64] or an anti-*Plasmodium* molecule such as SM1 [65]. Other bacteria such as *Asaia* spp and *Pantoea agglomerans* (formerly *Enterobacter agglomerans*) have also been used. Favia et al. showed that the *Asaia* associates stably with *An. stephensi* and that transgenic strain of this bacteria expressing GFP are able to colonize the gut and salivary gland of females of this mosquito [66]. In another study using *Asaia* spp, paternal transmission of recombinant strains expressing the green fluorescent protein GFP or the red fluorescent protein DsRed to progeny through mating of paratransgenic males with wild females was obtained in *An. stephensi* showing that it is possible to utilize nonbiting male mosquitoes in malaria transmission [67]. Working with *Pantoea agglomerans*, Wang et al. were able to express several anti-*Plasmodium* molecules such as SM1 peptide, scFv, mutated phospholipase (mPLA2), *Plasmodium* enolase-plasminogen interaction peptide (EPIP), synthetic antiparasitic lytic peptide Shiva1, etc., in both *An. gambiae* and *An. stephensi* and successfully suppressed transmission of *Plasmodium falciparum* and *P. berghei*, respectively [68].

Besides bacteria, fungi and viruses have also been utilized in mosquito paratransgenesis. The entomopathogenic fungi *Metarhizium anisopliae* was engineered by Fang et al. to express the anti-*Plasmodium* molecules SM1, a sporozoite-agglutinating scFv, as well as an antimicrobial toxin scorpine in *An. gambiae* [69]. Using the denonucleosis virus (DNV) in a proof-of-concept viral paratransgenesis work in *An. gambiae*, the potential of virions in paratransgenesis

was demonstrated by ability of transgenic DNV expressing GFP to infect larvae, persist to the adult stage and disperse to vital tissues such as fat bodies, ovaries and midgut, and be transmitted subsequently to other generations [70]. This shows that viruses such as DNV could be used to express antiparasitic molecules not only in one but several mosquito developmental stages and subsequent generations and could effectively mitigate and eliminate malaria transmission. Another virus, the Sindbis virus, has also been exploited in paratransgenesis and used to express scFv that acts against *Plasmodium gallinaceum* sporozoites in *Ae. aegypti* [71]. As it appears, the Sindbis virus has great potentials for control of various viruses transmitted by *Aedes* mosquitoes [72–74].

3.1.2. Sandflies

Efforts on control of sandfly vectors that transmit *Leishmania* parasites which cause the disease Leishmaniasis has been done mainly with chemical insecticides. To develop a more environment-friendly strategy, Hurwitz et al. recently demonstrated the feasibility of paratransgenesis for sandflies in a proof-of-principle work in *Phlebotomus argentipes* in which they used recombinant *Bacillus subtilis* fed to larvae to express GFP in the gut lumen of emerging adults (**Figure 5**) [75]. This proof-of-principle study has paved the way for future development of strains that will express anti-*Leishmania* molecules and block transmission of the parasites by the sandfly vector.

3.1.3. Tsetse flies

Although several control strategies including SIT have been applied against tsetse flies, continual effort is made to develop other methods that would not have limitations of the existing methods, be more sustainable, more cost-effective or suitable for use in IPM control. To investigate the possibility of paratransgenesis in tsetse flies, transgenic *Sodalis glossinidius* were introduced into adult females where they were able to express GFP and interestingly passed on subsequently to the progeny of those females [76, 77]. Actual utilization of antitrypanosomal molecules to block parasite development and transmission by tsetse flies could be achieved in the near future.

Figure 5. Paratransgenic sandfly *Phlebotomus argentipes*: (A) auto fluorescence of the outer carapace of the sand fly is seen amidst the presence of GFP expressed by the symbiont, (B) *subtilis* in the sand fly's midgut B. visualization of GFP specifically localized in the sand fly's midgut chamber upon uncoupling of the GFP signal from the background [75].

4. Advantages, disadvantages and limitations of transgenic and paratransgenic technologies

4.1. Transgenic technology

4.1.1. *Advantages of transgenic technology*

The main advantage of transgenic biotechnology is its ability to generate strains that possess traits that are unique and special, and accurately designed or tailored to be specific as desired. Also, the flexibility of transgenic technology allows generation of such desired strains in many species which would have been very difficult or impossible to achieve by other means. Transgenic strains are usually generated after one generation (**Figure 1**), and hence take less time to generate compared to other methods like classical genetics. Moreover, generation of strains possessing desired traits in one species can almost always be reproduced and transferred to related species with relative ease [78]. Quite unlike earlier genetic methods such as SIT where the use of radiation generates unknown and uncharacterized genetic mutations, transgenic technologies generate known and characterizable genetic modifications. Transgenes could easily be thoroughly characterized, and same goes for genomic positions in the dipteran insect where desired transgenes had got inserted. Also, most transgenic strategies are environment-friendly, sustainable and target-specific. For example, while chemicals developed against fruit flies may kill pollinator insects, transgenic strains developed for control of fruit flies are not likely to have any negative effect on pollinators that their wild counterparts do not already exhibit. Also, the development of resistance against control agents is less likely to occur when using transgenics.

In terms of costs, transgenic technologies as well as many other control strategies are not so cheap to develop. But it is difficult to say with all certainty whether transgenic (and paratransgenic technology) is cheaper than many other methods as there have not been any such economic studies to the best of our knowledge. Nevertheless, transgenic (and paratransgenic) approaches are considered less expensive with regards to the farmer or public beneficiaries as they are usually area-wide-oriented and implemented by big organizations at overall little or no cost to the individual farmers or the public.

4.1.2. *Disadvantages of transgenic technology*

Probably due to the fact that transgenic technology is just beginning to move from laboratory to the field [79, 80], there are yet no scientifically proven disadvantages. Despite this, many public negative concerns already exist on the use of transgenics [78], mostly environmental and social, as well as safety and ethical issues. These are mainly due to speculations and the uncertainty as to what might happen in nature following field use of transgenics, and whether unintentional and unforeseen mutations could lead to harmful consequences (though these can potentially occur also in nontransgenics). There are also thoughts on how field use of transgenics could interfere with diversity and evolution due to possible loss of genetic material of original insects and the associated future downstream events. Potential horizontal transfer of transgenes could also be a potential disadvantage that could be associated with the use of transgenic technology. However, a "self-limiting" transgenic approach such as use of

transgenic lethality dipteran strains should not present some of the afore-mentioned environmental problems since they are most likely to remove themselves from the environment with time unlike “self-sustaining” strains [81]. Though not always the case, there may be fitness costs that might arise in dipterans and other insects due to the various loads of transgenes they carry [82, 83]. When well assessed, the fitness costs could be determined and measures taken to eliminate them if necessary or avoid using strains that suffer such lack of fitness. New transgenic strains that may not have the observed fitness cost could also be developed and utilized instead. Proper assessment should be done to determine the associated risks and benefits before any GMO can be utilized [84].

4.1.3. Limitations of transgenic technology

Transposable elements or “jumping genes” have been the main tool relied on to achieve germline transformation and generate transgenic dipterans. However, most of the transposons used in dipterans (and other insects too) are insect-derived [85] and a major concern is that a transposon could potentially be remobilized from its integrated genomic position in the insect if transposases required for its activity is encountered in the field. The consequence of such transgene-transposase exposure could be the remobilization of a transgene to another genomic location or total loss of a transgene from an insect’s genome. Measures to avoid potential transgene remobilization in engineered dipterans such as postintegrational transgene modification to alter the transposon and achieve nonmobilization or stability has been demonstrated in *D. melanogaster* and *C. capitata* [86–88]. Other strategies that offer transgene stability are becoming available. The recently developed genome editing tool, CRISPR/Cas9, which allows RNA-guided modification of target DNA locations [41] has been utilized to achieve stable germline transformation in *D. melanogaster* [43]. Unlike transposon-mediated germline transformation, CRISPR/Cas9-mediated germline transformation is seamless and should not be prone to subsequent remobilization.

Transgenesis is not yet possible in all dipterans as not all members are amenable to it. Since the development of a transgenic insect strain involves germline transformation (**Figure 1**), it is therefore important that the biology of a target insect must be in such a way that allows the necessary manipulations to achieve genetic transformation. Tsetse flies are yet to be genetically transformed due to their viviparity which makes it difficult to obtain embryos needed for microinjections and subsequent germline transformation [77].

4.2. Paratransgenic technology

4.2.1. Advantages of paratransgenic technology

While similar to transgenic approach in terms of its ability to generate within a short time strains that possess unique and special traits designed specifically as desired, paratransgenesis also has an additional advantage of leaving the insect itself genetically unmodified and rather targets the parasites transmitted. This gives paratransgenic approaches a major plus in the sense that it has a more positive public perception than transgenic approaches as many of the disadvantages with use of transgenics would not be present [78, 89]. In addition, this technology has a high potential to be transferred between different species [78]. Moreover,

paratransgenic biotechnology mostly employs microorganisms that live within the target dipterans (symbionts) and as a result also has a high likelihood of field success. Another advantage of paratransgenics is the absence of fitness cost of genetic manipulation compared to transgenics or other control strategies [90].

4.2.2. Disadvantages of paratransgenic technology

Field application of paratransgenic strategies is yet to be actualized and any potential disadvantage of this technology is still to be proven scientifically. Nevertheless, safety concerns and risk assessments have become necessary requirements that need to be addressed to ensure that the benefits outweigh the risks of utilized genetically modified organisms [84]. One concern for paratransgenics is the potential exposure of engineered symbionts to the environment and likely consequences such as horizontal gene transfer. Measures such as symbiont encapsulation to ensure regulated release are being taken to address some of these regulatory concerns [91].

4.2.3. Limitations of paratransgenic technology

Despite the known advantages of the paratransgenic approach, a major limitation is that it is not suitable for most dipteran crop pests and has been developed mostly for those dipterans (and other insects) that transmit disease pathogens. Symbiont choice and utilization in a paratransgenic expression approach depend not only on availability of symbionts that can be isolated, cultured, reintroduced, and survive well in the targeted host, but also on the ability of the symbiont to be genetically transformed and to possibly express antiparasitic molecules [77]. The lack of some of these requirements would render several good symbionts unusable for paratransgenic control. The bacteria symbiont *Wolbachia* is one such microorganism that is promising for paratransgenic application, but the lack of success in genetically transforming it has hindered its further utilization for expression of antiparasitic molecules.

5. Future of transgenic and paratransgenic technologies

In the near future, transgenic and paratransgenic pest/vector control strategies may become common place and more widely applied than it is now. Some of the novel approaches of these technologies are promising and offer great hopes for control of several human diseases and could be implemented in the near future if regulatory and ethical issues are satisfied [92, 93]. This could usher in a new era where cases major dipteran-vectored diseases of man such as malaria and dengue, as well as agricultural pest like Tephritid fruit flies become much reduced or even eradicated.

The arrays of weapons in man's arsenal against his dipteran enemies are also expected to continue to expand. Continuous improvement will be made to existing control strategies, while new and better strategies are expected to be developed in the future as more advances are made in genetics and molecular biology. The RNA-guided genome editing tool, the CRISPR/Cas9 endonuclease recently developed from bacteria such as *Streptococcus pyogenes*

and *Neisseria meningitidis* [41, 94] has equally enabled genome modification and generation of transgenic control strategies in dipterans [43, 44, 50, 95]. More recently, a DNA-guided genome editing which makes use of an argonaute from the bacteria *Natronobacterium gregoryi* [96] has also been developed and it is expected that this new tool, as well as others that may soon be developed, will definitely lead to the generation of new transgenic or paratransgenic approaches to better control pest, nuisance or vector dipterans.

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