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A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly

Peng Gong[1], Matthew J Epton[1], Guoliang Fu[2], Sarah Scaife[2], Alexandra Hiscox[1], Kirsty C Condon[2], George C Condon[2], Neil I Morrison[1,2], David W Kelly[1,2,3], Tarig Dafa'alla[2], Paul G Coleman[2,3] & Luke Alphey[1,2]

The Sterile Insect Technique (SIT) used to control insect pests relies on the release of large numbers of radiation-sterilized insects. Irradiation can have a negative impact on the subsequent performance of the released insects[1–4] and therefore on the cost and effectiveness of a control program[5]. This and other problems associated with current SIT programs could be overcome by the use of recombinant DNA methods and molecular genetics[6–12]. Here we describe the construction of strains of the Mediterranean fruit fly (medfly) harboring a tetracycline-repressible transactivator (tTA) that causes lethality in early developmental stages of the heterozygous progeny but has little effect on the survival of the parental transgenic tTA insects. We show that these properties should prove advantageous for the implementation of insect pest control programs.

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is the world's most economically important agricultural pest insect, attacking more than 250 kinds of fruits, nuts and vegetables. In addition to the direct damage caused by infestation, many countries will not allow the importation of any produce that might harbor medfly. Medfly and other tephritid fruit flies are the target of large-scale suppression, eradication and preventative control programs based on SIT[13–15]. SIT depends on the mass rearing, sterilization and release of large numbers of insects. Released sterile insects compete for mates with wild insects. Mating to sterile insects reduces the reproductive potential of the wild population, and if sufficient sterile insects can be released for a sufficient period of time, the target population will crash and may even be locally eradicated. The paradigm for this approach is the complete elimination of the New World screwworm, *Cochliomyia hominivorax* (Coquerel), from North and Central America, and the successful prevention of its reinvasion from South America by regular release of sterile insects in a barrier zone in Panama[16].

SIT provides an environmentally friendly, species-specific method of pest control. It is a potentially attractive option for both suppression and elimination programs, and for preventative or remedial action against exotic pests[13,14,17]. However, despite some large-scale successes, SIT is used against only a rather modest range of pest species. Part of the problem is that wild-type strains of a pest insect may not be ideal for use in SIT. Properties such as a genetic marker to allow easy discrimination of released versus wild insects, and a system to avoid the need for radiation sterilization, would be highly desirable. Recombinant DNA technology can potentially provide these benefits and, if designed to be readily transferred between species, would allow SIT to fulfill its potential as an effective, environmentally friendly pest control system of broad applicability.

We attempted to develop a dominant lethal genetic system for use in medfly. Such a strain could be used as a redundant back-up for, or replacement of, sterilization by irradiation, either on its own or, preferably, in combination with the genetic sexing strains already constructed by classical genetics[18]. This is a version of a system known as RIDL (release of insects carrying a dominant lethal), prototypes of which have been constructed in *Drosophila melanogaster* using tTA[6,9,10,19]. We also provided a tightly linked genetic marker to allow discrimination of wild type and engineered insects ('RIDL insects'). Such a system should have minimal adverse impact on the released insects themselves, or on insects in the mass-rearing facility. This could potentially be achieved by the use of developmentally regulated promoters, inactive in adults, to drive expression of tTA, combined with a highly toxic effector under the transcriptional control of tTA, but with a very low basal expression to minimize the adverse effects in nontarget insects[10]. Unfortunately, the necessary molecular tools are not available in any pest insect.

Instead, we chose to simplify the system by using tTA as both the transactivator and the effector (**Fig. 1**). Low-level expression of tTA has been widely used in gene expression studies and is thought to be innocuous, whereas high-level expression of tTA is thought to be deleterious to cells, probably due to transcriptional 'squelching' and/or interference with ubiquitin-dependent proteolysis[19–23]. We therefore speculated that sufficiently high-level expression of tTA during development might be lethal.

Autoregulatory tTA systems have been used in transgenic mice with no obvious deleterious effects, despite showing the expected tetracycline-regulated expression of tTA[24]. Clearly, tTA does not accumulate to a lethal level in these mice. We constructed an expression cassette

[1]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK. [2]Oxitec Limited, 71 Milton Park, Oxford OX14 4RX, UK. [3]Department of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK. Correspondence and requests for materials should be addressed to L.A. (luke.alphey@zoo.ox.ac.uk).

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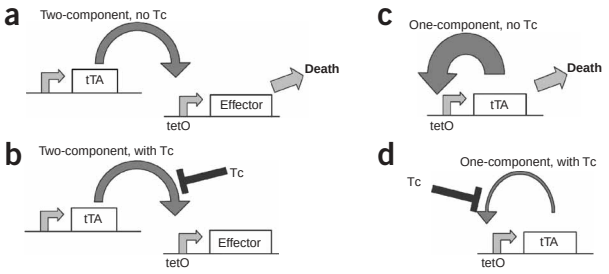


Figure 1 Tetracycline-repressible lethal systems. (**a**,**b**) Two-component system as previously published[9–11]. tTA[19] is placed under the control of a suitable promoter, for example, constitutive, female-specific, embryo-specific. In the absence of tetracycline (Tc) tTA binds tetO, driving expression of an effector molecule and leading, in the case of a lethal effector, to death (**a**). In the presence of tetracycline, tTA binds tetracycline; the tetracycline-bound form does not bind DNA, therefore does not activate expression of the effector, and the system is inactivated (**b**). (**c**,**d**) A simplified one-component system. In the absence of tetracycline, basal expression of tTA leads to the synthesis of more tTA, which accumulates to high level (**c**). This level can be regulated by modifying the stability and translational efficiency of the tTA mRNA. At the highest levels, expression is lethal, so tTA is both the driver and the effector. In the presence of tetracycline, tTA is inactivated by tetracycline and is therefore expressed only at basal levels (**d**). Tc, tetracycline.

intended to maximize derepressed expression of tTAV, which is a variant tTA sequence optimized for expression in *D. melanogaster*, while giving only low basal expression when exposed to tetracycline. In addition to giving repressible expression of tTAV, the compact design of these constructs (**Fig. 1c**,**d**) should also minimize the target for spontaneous mutation, and increase transformation efficiency. Medfly embryos were injected with either of two such constructs, LA656 and LA928, which differ in the details of the red fluorescent marker that each also includes. Three stable transgenic lines were recovered, each behaving as single autosomal elements inherited in a simple mendelian fashion. Heterozygous males from each line were crossed to virgin wild-type females, and their progeny reared either on a normal diet or on a diet supplemented with 100 μg/ml tetracycline ('tetracycline diet'). Whereas all genotypes survived well on the tetracycline diet, very few (0.2–0.7%) heterozygous transgenics survived on the non-tetracycline diet, relative to their wild-type siblings (**Fig. 2**). LA656 and LA928 therefore confer dominant lethality in the absence of dietary tetracycline, killing the affected individuals before eclosion, with most (92–98.7%) dying before puparium formation.

The few insects escaping the lethal effect of LA656 or LA928 in the absence of tetracycline are far too rare to compromise the effectiveness of a mass-release program. However, if this survival were heritable, in other words if there were a preexisting resistance factor in the wild population, this would tend to increase in frequency during such a program, which would quickly lose effectiveness. We investigated this possibility by mating the escapers to wild type, rearing the progeny on a nontetracycline diet, and again scoring the frequency of survival of transgenics relative to wild type. No significant increase in survival rate was observed relative to the previous generation (egg-to-adult survival on a nontetracycline diet: F_1, 0.7% ($n = 1855$); F_2, 0.5% ($n = 4590$); $\chi^2 = 1.18$; d.f. $= 1$, $P > 0.05$), indicating that survival is not determined by heritable factors. Nonetheless, the possibility of biochemical resistance to the lethal effector molecule remains a potential drawback to RIDL that is not a significant issue for radiation-based sterilization. Other forms of heritable resistance that apply equally to RIDL and SIT, such as assortative mating, have not proved to be a major problem for SIT programs to date.

Ideally, all affected individuals would die as embryos; this would minimize damage to the fruit from larval feeding, though oviposition damage ('stings') would still occur. The present lines do not achieve this, as a significant proportion of transgenic embryos hatch, though very few survive to pupate (**Fig. 2**). The importance of this varies by application: in a preventative release program with mixed-sex release, the additional damage might be unacceptable; this would be greatly mitigated by use of male-only release and might be considered insignificant in a suppression program targeting a preexisting wild population. Strains with earlier lethality could presumably be isolated by screening a large panel of insertion lines for this characteristic. The proportion of escapers and the time of death could presumably also be improved by constructing a line homozygous for more than one insertion. This would have the further advantage of providing a degree of redundancy against inactivation of one lethal construct, for example by random mutation. On the other hand, multiple homozygous lines would be harder to construct, especially where it is considered important to maintain genetic diversity within strains, and would presumably have lower fitness than either of the parent single insertion lines.

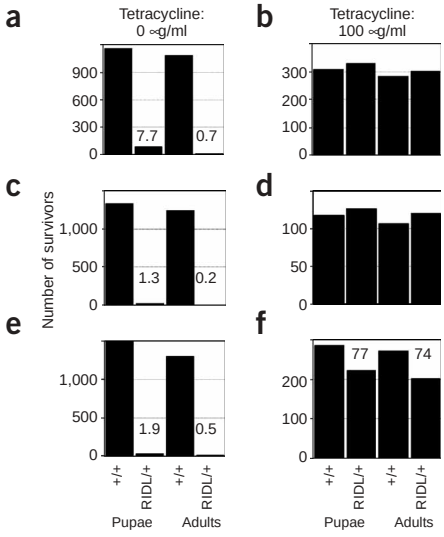


Figure 2 Repressible dominant lethality in transgenic medfly. Male medfly heterozygous for a one-component construct (see **Fig. 1c**,**d**) were crossed to virgin wild-type females. (**a**–**f**) Transgenic and nontransgenic progeny were reared together on a normal diet (nontetracycline diet, **a**,**c**,**e**) or on a diet supplemented with tetracycline to 100 μg/ml (tetracycline diet, **b**,**d**,**f**). In each panel, the number of progeny successfully pupating (column 1: wild type, column 2: transgenic), and successfully eclosing (column 3: wild type, column 4: transgenic) are plotted. If there were no differential mortality, equal numbers of each genotype would be expected to survive at each life stage. The absolute numbers of eggs used varied between experiments, being generally lower for the tetracycline diet; the key data are the relative survival of wild-type and transgenic individuals. Data for three independent lines are shown: LA656 (**a**,**b**), LA928f1 (**c**,**d**) and LA928m1 (**e**,**f**). In each case, highly significant dominant lethality, relative to wild type, was observed in transgenic insects raised on nontetracycline diet. The numbers above a column represent the percentage survival relative to wild type at each developmental stage, where this is significantly different from 100%. The three insertions are all >99% lethal in the absence of tetracycline; only LA928m1 shows a significant reduction in viability at both pupal and adult stages in the presence of 100 μg/ml tetracycline (two-tailed binomial test of unequal genotype frequencies, $P = 0.005$ and $P = 0.001$, respectively).

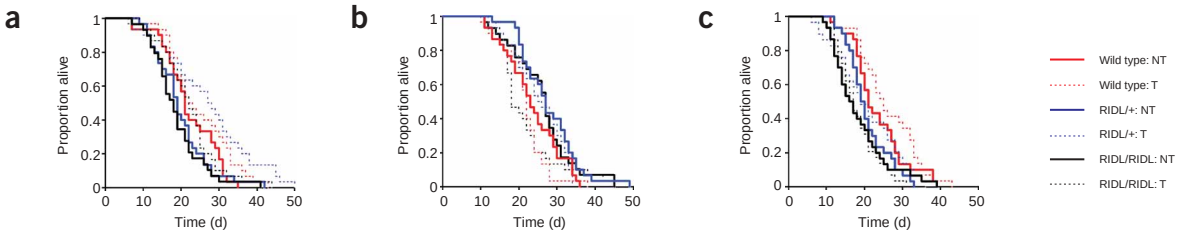


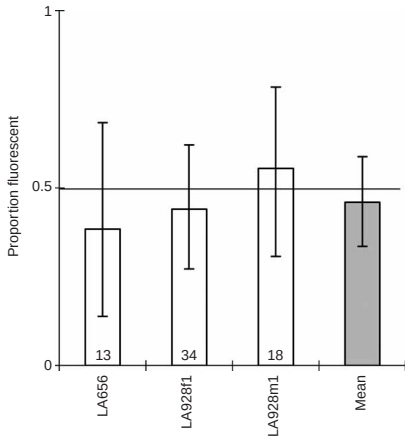
Figure 3 Effect of derepression of tTAV on adult male longevity. Heterozygous transgenic insects were allowed to mate. Their progeny were raised on a tetracycline diet, separated as pupae into their 3 genotypic classes (homozygous wild type, heterozygous transgenic or homozygous transgenic), then provided as adults either with a normal diet (NT) or a tetracycline diet (T). Data are the sum of three replica experiments, each with ten males. In the rare instance when an adult escaped or was killed during the daily counting of survivors, such an adult was excluded from the analysis. LA656 males have a similar mean lifespan to wild type in the presence of tetracycline (22.1 d); without tetracycline this is reduced by 13% (19.3 d). LA928Am1 showed a tetracycline-independent reduction of 21% (wild type 24.7 d, transgenic 19.3 d). In each case homozygotes appeared to do slightly worse than heterozygotes, but these differences were not statistically significant. LA928f1 showed no significant reduction in mean lifespan relative to wild type.

For a SIT-like pest control program, it is essential that the released adults be reasonably long lived and competitive. However, released adults would no longer receive dietary tetracycline. We therefore investigated whether LA656 and LA928 are lethal to adults deprived of tetracycline post-eclosion. Heterozygous transgenic medfly were allowed to mate; their progeny were raised on a diet supplemented with tetracycline. After eclosion, adults were maintained on a tetracycline diet or transferred to a diet lacking tetracycline. The daily survivorship of adult males of each of the three genotypic classes (homozygous transgenic, heterozygous transgenic and homozygous wild type) was determined. The transgenic lines showed good adult survival relative to nontransgenic controls (**Fig. 3**). Modest reductions in mean lifespan were seen for two of the lines; in one case this effect was tetracycline-repressible, implying that it was an effect of elevated tTAV, possibly through ectopic expression of a nearby gene. In the other case it was not tetracycline-repressible, implying that the reduction in lifespan was due to insertional mutagenesis or expression of the marker, rather than expression of tTAV. Only one line, LA928m1, showed a substantial reduction in egg-to-adult survival of heterozygotes in the presence of tetracycline, relative to wild-type controls (**Fig. 2f**); this line also showed a substantial, tetracycline-independent reduction in lifespan relative to wild type (**Fig. 3c**). LA928f1 showed no significant reduction in lifespan relative to wild type (**Fig. 3b**, log-rank comparison of survival functions, $\chi^2 = 1.68$, d.f. $= 1$, $P > 0.05$), while giving the highest percentage mortality for larvae reared in the absence of tetracycline (**Fig. 2c**). This line-to-line variation indicates that deleterious effects are not an inherent property of the design of these constructs, and that optimal lines could be selected by applying these straightforward assays to a large panel of insertion lines.

We also analyzed the mating competitiveness of the transgenic males. We introduced wild-type virgin females into a mating arena containing five homozygous transgenic males and five wild-type males, to simulate the natural lek-based mating system. No significant reduction in competitive mating ability relative to wild type was observed for any of the transgenic lines, indicating that competitive RIDL strains can be generated efficiently (**Fig. 4**).

Derepression of tTAV expression seems to have a rather modest effect on adults compared with the lethality seen in larvae and pupae. This might be because adults are relatively tolerant of high levels of tTAV, or because tTAV does not accumulate in adults, perhaps as a consequence of residual tetracycline retained from their exposure as larvae. We therefore assessed the expression of tTAV in LA656 adults and larvae, using quantitative RT-PCR. We found that tTAV expression is strongly elevated in adults kept on a nontetracycline diet for only 2 d, particularly in homozygotes (48-fold increase over basal expression in heterozygotes, 672-fold in homozygotes, **Supplementary Table 1** online). We conclude that the autoregulatory tTAV expression system is functional in adults as well as larvae, that exposure to dietary tetracycline at 100 μg/ml throughout larval life does not contribute repressive levels of tetracycline that persist long into adulthood, and that high-level expression of tTAV is much less deleterious to adults than to earlier developmental stages. We speculate that either transcriptional squelching or interference with ubiquitin-dependent protein degradation may be less disruptive to fully formed adults than

Figure 4 Effect of derepression of tTAV on adult male mating competitiveness. Five wild-type females were introduced into a mating arena containing five homozygous transgenic males and five wild-type males. All males were 5-d-old virgins. After 6 h, a time insufficient for repeat mating, the females were removed. The genotype of the mating partner was inferred by analysis of the progeny: a female who had mated a transgenic male would produce fluorescent progeny; a female who had mated a wild-type male would produce nonfluorescent progeny. The first 3 bars show the mean and 95% confidence intervals for mating success of transgenic males of each line; the 4th bar (shaded grey) represents the combined data from all 3 lines. The number of females tested and giving progeny is shown at the foot of each bar. If transgenics and wild type were equally competitive, then the proportion of females that gave fluorescent progeny would tend to 0.5. Within these relatively small-scale laboratory experiments we found no significant difference between any of the lines and 0.5, nor any significant difference between any one line and another. The mean of the proportion fluorescent was very close to 0.5 at 0.46 (95% CI = 0.34 – 0.59), indicating that lines of sexually competitive RIDL medfly can readily be generated.



to earlier stages, which must carry out a developmental program that relies on precise temporal and spatial control of the expression of many different genes.

A potential risk associated with current SIT programs is that the mass-rearing facility itself holds a large number of dangerous pest insects. Radiation-sterilization is an 'inducible' process, in that the insects are fertile unless and until they are adequately irradiated. Nonirradiated releases, due to accident or sabotage, could be highly damaging, especially in a region otherwise free of the pest. In fact SIT has an excellent safety record in this respect, but such releases have indeed occurred, for example, nonirradiated New World screwworm were released in Mexico in 2003 (ref. 25). The strains described here, in contrast, have a repressible lethal system, so that they are kept alive by a dietary additive but they or their progeny will die in the wild without any further action or process being required. RIDL therefore provides a fail-safe aspect lacking in current programs, though the use of genetic sexing strains, for example, for medfly[18], may also mitigate the harm caused by a nonirradiated release, depending on the nature of the incident.

We have shown that highly efficient, repressible, dominant lethality can be achieved with compact constructs in a major pest species, giving performance characteristics appropriate for incorporation into SIT-based control programs. These constructs also provide a convenient genetic marker. Repressible dominant lethality potentially provides a replacement or redundant back-up for radiation-sterilization; the requirement for a dietary additive for larval viability would give a 'fail-safe' aspect that would mitigate any failure in the irradiation procedure. Since these constructs contain no tephritid DNA, we expect them to work across a wide phylogenetic range; this technology should therefore be much easier to transfer to other species than are the products of classical genetics.

METHODS

Medfly transformation and rearing. Medfly transformation with transposons LA656 and LA928 was done using standard microinjection-based methods. Medfly were reared on a standard yeast-wheatgerm-glucose-agar *D. melanogaster* diet, supplemented as appropriate with tetracycline hydrochloride (Sigma-Aldrich) to 100 μg/ml, at which concentration tetracycline appears to have no adverse effect. Heterozygotes and homozygotes were differentiated on the basis of fluorescence intensity (see **Supplementary Fig. 1** online). Homozygous stocks of each line have been continuously maintained on this diet for up to 20 generations. Each insertion was derived from independent groups of G_0 parents and behaves as a single, autosomal element segregating in a mendelian fashion (**Supplementary Table 2** online).

Plasmids pLA656 and pLA928. Plasmids pLA656 and pLA928, containing *piggyBac*-based transposons LA656 and LA928, respectively, are shown in **Supplementary Figs. 2–4** online. These two plasmids differ primarily in the promoter used to control expression of the DsRed2 (Clontech) fluorescent marker protein. For LA656 this is a fragment of the *ubi-p63E* polyubiquitin gene of *D. melanogaster*; for LA928 it is a fragment of the *immediate-early-1* (*ie1*) gene of the *Ac*MNPV baculovirus, together with the hr5 enhancer from the same baculovirus.

Real-time PCR. Pools of three flies were homogenized in Trizol (Invitrogen); RNA was purified from the homogenates according to the manufacturer's instructions. For the RT-PCR we used Taqman chemistry and reagents (ABI), and an ABI Prism 7000 instrument. Each sample was assayed in triplicate; data are the mean of these three assays. tTAV expression was normalized to an 18S rRNA internal control. Probes were tTAV: VIC-TCGATCTGGACATGTTGG MGB, 18S: 6-Fam-CCGTCGTAAGACTAAC-MGB. Primers were tTAV: 5′-CAT GCCGACGCGCTAGA-3′ and 5′-GTAAACATCTGCTCAAACTCGAAGTC-3′; 18S RNA: 5′-ACGCGAGAGGTGAAATTCTTG-3′ and 5′-GAAAACATCTTTG GCAAATGCTT-3′.

Accession number. The accession number for the tTAV sequence is AJ865387.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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