Engineering Insects for the Sterile Insect Technique

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ABSTRACT The mass-release of sterile insects (the sterile insect technique (SIT)) is a highly effective component of area-wide methods of pest control with no environmental impact. The SIT relies on the sterilization of large numbers of insects, usually by irradiation. The SIT has been used successfully against several pest insects. However, modern biotechnology could potentially provide several improvements. These include: (1) improving the identification of released individuals, (2) removing the need for radiation-sterilization, (3) reducing the hazard posed by non-irradiated accidental releases from the mass-rearing facility, and (4) providing automated sex-separation prior to release (genetic sexing). None of these are necessarily unattainable by classical methods. However, the use of recombinant DNA methods may allow these benefits to be obtained in a shorter period and to be transferred more readily from one species to another than are the products of classical genetics. The potential of these methods, and the progress towards realizing this potential, is discussed.

KEY WORDS genetic sexing, transformation, genetic sterility, marking, stability, fitness

1. Introduction

The sterile insect technique (SIT) has been used successfully against several insect species (Lindquist et al. 1992, Krafsur 1998, Tan 2000, Wyss 2000, Koyama et al. 2004, and other papers in this volume). However, modern biotechnology could potentially provide several improvements in the operation or security of a pest control programme integrating the SIT (Heinrich and Scott 2000, Thomas et al. 2000, Alphey 2002, Handler 2002, Benedict and Robinson 2003, Horn and Wimmer 2003, Gould and Schliekelman 2004). These include: (1) improving the identification of released individuals by providing a genetic marker allowing easy discrimination between wild-type and released insects, (2) removing the need for radiation-sterilization by providing some sort of "genetic sterilization", (3) reducing the hazard posed by non-irradiated accidental releases from the mass-rearing facility by arranging that the insects need an artificially-provided condition, for example a dietary supplement, in order to survive or reproduce, and (4) providing automated sex separation prior to release to eliminate females from the released population (genetic sexing). None of these is necessarily unattainable by classical methods. However, the use of recombinant DNA methods may allow these benefits to be obtained in a shorter period and to be transferred more readily from one pest species to another than are the products of classical genetics.

2. Genetic Markers

It is essential to be able to detect the presence of wild insects, even in the presence of overwhelming numbers of released sterile insects. This requires that the released insects be marked in some way to distinguish them from wild insects. This has been done by adding a

M.J.B. Vreysen, A.S. Robinson and J. Hendrichs (eds.), Area-Wide Control of Insect Pests, 51–60.

dye to their food, or dusting the pupae or adult insects with a fluorescent dye (Hagler and Jackson 2001). Provision of a suitable genetic marker in the strain would obviate the need for such a dye. This would reduce the amount of handling required, and the possibilities for human error. A candidate marker, *Sergeant* (*Sr2*) has recently been described for the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), which has a dominant effect on adult cuticle pigmentation (Niyazi et al. 2005). This mutation is also a recessive lethal. This would normally prevent the production of a true-breeding strain, but this problem can be avoided in this case due to the unusual genetics of Mediterranean fruit fly genetic sexing strains constructed at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria (Robinson 2002, Niyazi et al. 2005).

An alternative would be to provide a dominant marker by transgenesis, for example a gene expressing a fluorescent protein. Such systems have been widely used in *Drosophila* and pest insects, including several tephritids, mosquitoes and moths (Berghammer et al. 1999, Catteruccia et al. 2000, Peloquin et al. 2000, Pinkerton et al. 2000, Tamura et al. 2000, Handler and Harrell 2001, Horn et al. 2002, Perera et al. 2002, Allen et al. 2004a). In contrast to classical mutagenesis, dominant markers can readily be generated by this method and these markers are not associated with recessive lethality. It is clear that such markers can be provided in most species of interest for the SIT. Two recent papers (Catteruccia et al. 2003, Irvin et al. 2004) have shown that specific strains carrying such markers showed a severe reduction in fitness relative to the untransformed progenitor strain. However, in these cases the loss of fitness appears to have been due to inbreeding depression; when this was avoided, no such loss of fitness was observed (Allen et al. 2004b, Moreira et al. 2004).

An additional possibility would be to label sperm. It would be desirable to be able to determine whether a trapped female had mated a wild or a sterile male before she was captured. Different programmatic responses might be desirable for each of these possibilities, so accurate diagnosis is important. However, a quicker and more convenient option would be simply to look at the sperm, if a suitable fluorescent marker could be provided. Such marked sperm have been produced in *Drosophila* and used to monitor sperm storage and competition in laboratory experiments (Civetta 1999). This has also recently been achieved in the mosquito *Anopheles stephensi* Liston (Catteruccia et al. 2005), using a β2-tubulin promoter from *Anopheles gambiae* Giles to give expression in the male germ-line. These authors further demonstrated that automated sex separation could be achieved using the sex-limited expression of a fluorescent protein in the larval testis and a Complex Object Parametric Analyser and Sorter (COPAS) – a sorter based on fluorescence.

3. Genetic Sterilization

Ionizing radiation damages insects and can thereby have a significant negative impact on their subsequent performance (Shelly et al. 1994, Lance et al. 2000, Barry et al. 2003, Kraaijeveld and Chapman 2004), and therefore on the cost and effectiveness of the sterile insect release, although the exact magnitude of this effect is still controversial (Robinson et al. 2004). Irradiated male Mediterranean fruit flies compete less well for mates, and are less effective at inducing female refractoriness to remating (Kraaijeveld and Chapman 2004). In some cases, irradiated insects also have a reduced lifespan after release relative to wild insects. This further reduces their effectiveness. However this reduction in lifespan is probably not entirely due to radiation, other possible contributory factors being damage due to handling and distribution, and the conditions and genetic pressures of mass-rearing.

Radiation works in this context by inducing random dominant lethal mutations in the gametes of irradiated insects. Progeny from such gametes therefore die, typically early in

embryogenesis. It should be possible, in principle, to remove the need for irradiation by using engineered dominant lethals instead (Fryxell and Miller 1995, Alphey 2002, Alphey and Andreasen 2002). Strains with the necessary properties were constructed in *Drosophila* some years ago (Heinrich and Scott 2000, Thomas et al. 2000, Horn and Wimmer 2003). Much more recently, the construction of potentially suitable strains of Mediterranean fruit fly has been described (Gong et al. 2005). These, the first such strains described for any pest insect, contain a novel, single-component, repressible, dominant, lethal gene which gives up to 99.8% mortality in heterozygotes not provided with dietary tetracycline. Penetrance of lethality and effect on adult male lifespan varied, as expected, from one insertion line to another – this is the well-known phenomenon of position effect. However, the best line showed 99.8% lethality in the absence of tetracycline, no effect on adult male lifespan even in the absence of tetracycline, and no detectable negative effect on male mating competitiveness. The effect on female refractoriness to remating was not measured.

Along with the major benefit of eliminating the requirement for irradiation, and thereby the expense, security issues, and damage to the insects associated with this, there are some potential drawbacks with this approach, and with the particular strains described by Gong and colleagues (2005). Mass-release of genetically engineered insects, even of this particularly benign type, will face regulatory hurdles that have not been imposed on the use of irradiation and the products of classical genetics. The present strains require the addition of tetracycline, or a suitable analogue, to the larval diet. Such antibiotics are not normally used in Mediterranean fruit fly mass-rearing, but chlortetracycline is a component of the standard diet used for mass-rearing pink bollworm. It is also a component of the liquid diets being developed for Mediterranean fruit fly mass-rearing by the United States Department of Agriculture's Agricultural Research Service (USDA-ARS), so the use of tetracyclines seems not to be an insuperable problem. Another issue is the use of transposons as gene vectors. Consideration of the mechanisms and rates of horizontal gene transfer, and of its consequences in this context, clearly lead the writer to conclude that this is not a serious issue for non-autonomous transposons that contain no sequences likely to give a selective advantage to the recipient, and no functional selfish DNA or gene-drive systems.

However, some commentators believe otherwise, and this will doubtless be one of several issues that regulatory authorities will need to consider. In any case, this is not an insuperable issue, as systems are being developed to stabilize transposable elements after insertion (Handler et al. 2004, Dafa'alla et al. 2006, Handler, this volume). The strains described by Gong et al. (2005) have a larval lethal phase, rather than the (preferred) embryonic lethality caused by irradiation. This could be a problem for some bisexual releases; in such cases efforts would need to be made to develop a transgene system with an earlier lethal phase (Horn and Wimmer 2003), or a genetic sexing system.

Perhaps the only non-trivial drawback of repressible lethals as a replacement for radiation sterilization is the question of resistance management. Though heritable resistance to the SIT is possible, for example by assortative mating, in which the wild females preferentially mate wild males rather than sterile males, in the 50-year history of the SIT there has been very little evidence of this (but see Koyama et al. 2004 for one clear example). The use of engineered repressible lethals would open up another possible form of resistance, namely "biochemical" resistance to the lethal effector molecule of the engineered system. This is essentially impossible for radiation-based SIT, as the random nature of the radiation damage means that every sperm is damaged in a different way, and it seems inconceivable that the wild population could evolve a mechanism to overcome this random damage. Whether such resistance could arise in practice, and over what

timescale, is entirely a matter of speculation at present. This possibility of resistance is not a new issue, and applies equally to the other major pest control methods of chemical pesticides and genetically engineered insecticidal crops. In each case, the evolution and spread of resistance can be managed or mitigated by various methods, including "stacking" effector molecules, etc. These methods can also be applied to the use of engineered repressible lethals.

Although only published so far for the Mediterranean fruit fly, similar systems are under development in the author's laboratory for several other pest species. In this context, it is important to note that the constructs of Gong et al. (2005) contained no Mediterranean fruit fly DNA, and might therefore be expected to work in a range of species with little or no modification.

4. Reduced Escape Hazard

Insect pest control programmes with an SIT component mass-rear the pest insect on a massive scale. Until these insects have been irradiated, any escape would be unwelcome, and potentially catastrophic. In fact, SIT applications have an excellent safety record in this respect, but non-irradiated releases have indeed occasionally happened, for example of New World screwworm in Mexico and Panama in 2003 (del Valle 2003). However, all accidental outbreaks were quickly eliminated by the release of additional sterile insects. Natural disasters (e.g. hurricanes or earthquakes), accidents or sabotage could also have very severe consequences. The use of genetic sexing strains for Mediterranean fruit fly SIT, such as the strains based on the *temperature sensitive lethal* (*tsl*) mutation mitigates these consequences as colony females are temperature sensitive. However, the engineered repressible lethal strains described above have the potential to provide a more satisfactory solution. These insects, or their progeny, will die unless they are artificially provided with an antidote to the lethal genetic system. This neatly overcomes the escape hazard, as such strains can neither establish in the wild on their own, nor form viable hybrids with any pre-existing wild pest population.

For this application, one would envision introducing an engineered repressible lethal genetic system into a strain currently used for the SIT, either a sexing strain or wild type, depending on what is available in the species of interest. It would also be simple to arrange that the engineered lethal system is associated with a useful genetic marker. This strain would then be used exactly as at present, but with the advantages of the genetic marker and of a dramatically reduced escape hazard.

The question of resistance, discussed above, does not apply to this approach as radiation would still be the primary basis of sterility, with the engineered system being a backup. This principle could still allow the use of a reduced radiation dose, relying on the engineered system to kill the few insects that would otherwise have been produced.

5. Genetic Sexing

For many insects, it would be highly preferable to eliminate females from the release population. This is for several reasons: (1) the females of some species are damaging while the males are not, (2) released males may court co-released sterile females, if present, rather than seeking out wild females, (3) the presence of females may require the use of a higher radiation dose than would be optimal for males, and (4) even if the females are merely neutral to the programme, they consume diet and add to distribution costs. Highly effective genetic sexing strains have been produced for various species, based on translocation of a dominant selectable marker to the Y chromosome (Whitten 1969, Whitten and Foster 1975, Seawright et al. 1978, Robinson 1989, Hendrichs et al. 1995, Franz et al. 1997, Robinson et al. 1999, Robinson 2002). Unfortunately, these chromosome aberrations that are an integral part of selection systems tend to reduce the rearing productivity of the flies that carry them. In Mediterranean fruit flies, these aberrations are also unstable (Franz et al. 1994, Kerremans

and Franz, 1995) but adequate strain quality can be maintained during large scale mass-rearing through the use of a filter rearing system (Fisher and Cáceres 2000). Furthermore, each of these sexing strains must be developed anew for each new species – genetic tools developed in one species by classical mutagenesis cannot be transferred to another species.

Recombinant DNA methods offer the prospect of simpler systems for genetic sexing, in which a repressible or inducible female-specific lethal is used. Such constructs could be introduced into an otherwise wild-type genetic background. Changing from permissive to repressive conditions in the last generation of mass-rearing would provide a single-sex population for release. For this purpose, the repressive condition could be high or low temperature (as for the present *tsl* strains), presence or absence of a dietary chemical, or any other convenient environmental parameter that can be tied to a change in gene expression or function. Such systems have been demonstrated in *Drosophila* (Heinrich and Scott 2000, Thomas et al. 2000), in Mediterranean fruit fly (Fu et al. 2007), and work is in progress to develop them for pest insects.

The *tsl*-based genetic sexing strains (e.g. VIENNA 8) developed at the FAO/IAEA Agriculture and Biotechnology Laboratory for the Mediterranean fruit fly, are extremely effective, despite some modest drawbacks. The greatest advantage of transgene-based sexing systems, relative to current practice, may therefore be seen in other pest insects, for which no such system is presently available. Development of a sexing system would have significant benefits for several species, including the New World screwworm *Cochliomyia hominivorax* (Coquerel), various fruit flies (e.g. the Mexican fruit fly *Anastrepha ludens* (Loew)), and anopheline mosquitoes, and has also been advocated for some moth species (Marec et al. 2005).

6. Disadvantages of Recombinant DNA Methods

Apart from the specific issues discussed above (use of tetracycline, possibility of resistance to a dominant lethal-based genetic sterilization method), two more general issues have been raised as possible limitations to the use of recombinant DNA methods for the purposes described above. These are fitness and stability.

6.1. Fitness

Two recent papers (Catteruccia et al. 2003, Irvin et al. 2004) have appeared to imply that all transgenic mosquitoes will have severely reduced fitness or mating competitiveness, compromising any genetic control strategy based on such insects. In fact these studies used a small number of highly inbred lines, and much of the fitness cost may be attributed to this inbreeding. Furthermore, even if these lines were shown to have low fitness, it does not follow that this will be true for all transgenic strains. A more recent study that avoided inbreeding found no significant deleterious effect in insects expressing a synthetic peptide as well as a fluorescent marker protein (Moreira et al. 2004). This is consistent with a much larger study of transgenic *Drosophila*, which found only modest effects on fitness in many strains (Lyman et al. 1996).

6.2. Stability

This relates to the use of non-autonomous transposable elements as transformation vectors. There is some debate over the stability and environmental safety of current transformation technology, which is based on the use of these transposons. The writer does not agree that the use of large non-autonomous transposons automatically results in an unacceptable risk. This is particularly clear when, as in the applications described above, the transposon contains no components that might confer a selectable advantage on a recipient, i.e. no insecticide or antibiotic resistance genes. Compared to the use of genetically-modified crops that (1) generally incorporate antibiotic resistance, herbicide tolerance and/or insect toxicity (from *Bacillus*

thuringiensis (de Barjac)), (2) liberate pollen into the environment, and (3) can hybridize with wild relatives far more readily (e.g. canola), the issue does not seem daunting. Furthermore, technical progress in this area may overcome or bypass this difficulty. There are several published methods, or obvious variants thereof, that would do this, though demonstrated only in *Drosophila* so far (Rong and Golic 2000, Groth et al. 2004, Handler et al. 2004, Horn and Handler 2005, Oberstein et al. 2005, Wimmer 2005), apart from the phage ϕC31 system, which was also shown to work efficiently in a mosquito (Nimmo et al. 2006). More generally, no strain or genetic construct is truly stable. All are subject to random mutation. This will lead, at a very low frequency of perhaps 10^{-7}-10^{-8} per insect generation, to the production of defective versions of the transgenic strain, particularly ones that have lost the intended function. The presence of such defective versions arising in the release generation should be entirely inconsequential, as they would be vastly outnumbered by correctly functioning insects. If the original transgene system was mildly deleterious, revertants arising within the breeding population of the mass-rearing facility might have a modest selective advantage over the original transgenic strain. In this case the defective version would tend to spread through the breeding population, which would be highly undesirable. However, this is not a new problem. The current translocation-based Mediterranean fruit fly sexing strains are also unstable. Since the males are semi-sterile and the females weakened by the recessive mutations they carry, breakdown products, or wild-type chromosomes introduced into the facility from outside, will spread rapidly through the mass-rearing population. This problem was overcome by introducing an elegant filter rearing system, in which a relatively small population is carefully maintained and monitored to ensure its genetic quality (Fisher and Cáceres 2000). Samples from this colony are taken, expanded for several generations and then released; no insects from this much larger population are used for subsequent breeding. This system is likely to be required for large-scale rearing of any non wild-type strain, whether produced by classical or molecular genetics. It should be more than capable of handling the level of instability that might be expected from a typical transgenic strain, although actual mass-rearing will be required to address this experimentally.

7. Combining the Benefits of Recombinant DNA Methods

The various improvements to the SIT discussed above could be provided independently, but it would be more efficient to combine them. For example, while there may be several ways to construct a genetic sexing strain, the use of a repressible system, where the repressor is not found in the natural environment of the insect, would have the additional advantage that escaped females, or female progeny, would die in the wild. The escape hazard would thereby be greatly reduced, which would not typically be true of inducible lethal systems, although they might give strains that were perfectly adequate for the purpose of genetic sexing. Furthermore, such a strain might also be used to remove the need for irradiation. Elimination of all female progeny will generally be as effective as killing all the progeny. Indeed it may be significantly better, particularly if several unlinked female-lethal constructs are combined in the same strain (Schliekelman and Gould 2000, Thomas et al. 2000).

It therefore seems likely that it will be possible in the near future to combine all of the above advantages – genetic marker, genetic sterilization, reduced escape hazard, and genetic sexing – into a single compact construct or genetic system. The construction of an effective non-sex-specific lethal system in Mediterranean fruit fly (Gong et al. 2005) is a major step towards this goal.

8. Conclusions

Recombinant DNA methods show great potential for improving SIT. Each of the

improvements discussed above, incorporated singly or in combination into a field programme, could provide substantial benefits (Simmons et al., this volume), and future advances in molecular biology and genetics are likely to allow further improvements as yet unheralded. Strains with sufficient potential to warrant incorporation into a field programme are already available, though some field-testing is urgently required to confirm this potential. The first few strains may well not perform in the field exactly as they do in the laboratory, and some modification of both strains and testing methods may be required. Even if the strains themselves are found not to need any further refinement, some adjustments may be necessary in the mass-rearing methods to incorporate these new genetic strains, as was required during the change to the current Mediterranean fruit fly sexing strains.

A more serious limitation to progress is the lack of a well-developed regulatory process to oversee the introduction of transgenic strains into field use. It is essential that the field use of recombinant DNA methods is subjected to appropriate expert assessment. Developments in the laboratory have not yet been matched by parallel developments in the regulatory process. However, some progress has been made, particularly in the USA. It is essential that countries that have an interest in the SIT urgently clarify and update their regulatory procedures, so that the potential of this new approach can be realized in the field.

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