DEVELOPMENT AND APPLICATION OF GENETIC SEXING SYSTEMS FOR THE MEDITERRANEAN FRUIT FLY BASED ON A TEMPERATURE SENSITIVE LETHAL MUTATION

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Abstract

DEVELOPMENT AND APPLICATION OF GENETIC SEXING SYSTEMS FOR THE MEDITERRANEAN FRUIT FLY BASED ON A TEMPERATURE SENSITIVE LETHAL MUTATION.

The present status in genetic sexing for the Mediterranean fruit fly is discussed. This includes the selection of the appropriate sexing gene (which determines the feasibility and practical applicability of the sexing system) as well as the selection of the appropriate Y–autosome translocation (which determines the stability of the sexing system). A temperature sensitive lethal mutation is used to eliminate females during the egg stage. This mutation in combination with new Y–autosome translocations allowed the construction of a genetic sexing strain, named VIENNA-42, that is stable enough for large scale mass rearing. Also described are the analysis of this strain under field cage and field conditions and, in preparation for large scale tests in Guatemala, the outcrossing of VIENNA-42 with genetic material from the target area.

1. INTRODUCTION

The sterile insect technique (SIT) is an environmentally friendly method for the control of pest insects. It has been applied successfully on numerous occasions and the number of ongoing or planned programmes increases constantly [1]. For the

medfly 12 mass rearing factories are in operation worldwide [2]. However, in all cases males and females are produced despite the fact that only the released sterile males contribute to the population suppression. The availability of a technique that allows the large scale elimination of females and, therefore, the release of only male flies can improve the SIT considerably in the following ways:

(a) *Reduction in programme costs*

— Reduced production costs: only the active agent is reared, transported and released.
— Simplified monitoring: trapped females can be used as direct indicators for the progress of the programme.

(b) *Increased efficiency*

— No assortative mating among the released flies and better dispersal of the flies in the target area.
— Improved male quality: in contrast to females, male pupae can be irradiated later, i.e. at an age where the radiation is less damaging to the overall viability of the fly.
— More flexibility in the timing of the release: in the absence of females, males can be released later, i.e. closer to their sexual maturity.

(c) *Increased application range*

— The SIT can be applied also in fruit exporting regions as the problems associated with sterile stings no longer exist (e.g. infection with pathogenic bacteria or fungi and transmission of these pathogens by females). As a consequence, application of the SIT is no longer restricted to eradication, but can also be used as a bioinsecticide to control the target population.

We describe the construction of sexing strains utilizing a temperature sensitive lethal mutation which allows elimination of the females at the egg stage. The genetic stability of these strains is primarily dependent on the structure of the Y–autosome translocation. Through genetic and cytogenetic analyses it was possible to develop strains with sufficient stability. Furthermore, some of these strains have been mass reared and evaluated under field cage and field conditions to assess their survival, dispersal and mating behaviour. Recently, one of these strains was outcrossed with genetic material from Guatemala in preparation for a larger scale mass rearing/field test in Guatemala. The aim is to produce 20 million males per week which will then be evaluated for their performance in the field.

2. RESULTS

2.1. Temperature sensitive lethal (*tsl*) mutation

By comparing and evaluating the existing selectable markers, it became apparent that none of them was suitable for a large scale mass production facility. In some cases, sexing would be too expensive (requiring expensive machines or chemicals), would not be accurate enough, would be allowed only at very late stages (no savings on larval diet) or would require the application of toxic chemicals. On the basis of these arguments it was suggested that the use of temperature inducible lethal mutations be considered instead. Within this group of lethal mutations it should be possible to identify those where lethality can be induced during early developmental stages, ideally during the egg stage. This stage is not only the earliest stage possible to eliminate the females, but it is also a very convenient stage to apply temperature as the eggs are maintained at high density in plastic containers.

To induce a temperature sensitive lethal (*tsl*) mutation, flies were treated with ethylmethane sulphonate (EMS), followed by the isolation of the treated chromosomes via single-pair mating steps. The treated chromosomes were made homozygous and each family was tested for temperature dependent lethality by incubating eggs at various temperatures and by recording the resulting egg hatch. In this large screen, one *tsl* mutation was recovered (G. Franz and E. Busch-Petersen, in preparation). In addition to the *tsl* mutation, this strain also carries a white pupa (*wp*) mutation. It turned out later that this is a very useful feature for the analysis as well as the practical application of the otherwise 'invisible' *tsl* mutation as it provides visual cues at the pre-release stage to whether the temperature treatment has been successful or not. This argument can be taken even further. Any new *tsl* based sexing system that is going to be isolated in the medfly, or any other fruit fly species, should contain a morphological mutation that is closely linked to the respective *tsl* mutation.

In a first series of tests we have shown that it is indeed possible to use the *tsl* mutation as a selectable marker when the mutation is combined with an appropriate Y–autosome translocation (G. Franz and E. Busch-Petersen, in preparation). In our tests, eggs were collected for 24 h, followed by a 24 h treatment with temperatures between 31 and 35°C. At 34°C all homozygous *tsl* genotypes are eliminated during very early stages, i.e. the lethality is visible primarily as reduced egg hatch (G. Franz, unpublished results).

Sublethal temperatures, e.g. 29°C, lead to a significant delay in development if the treatment period is extended to 48 h and longer. This effect leads to a separation of male (wild type) and female (*tsl*) pupation which can be used for sexing, though incomplete.

In addition to the initial tests mentioned above, the *tsl* mutation was analysed with respect to its genetic behaviour. If homozygous *tsl* females are crossed with wild

type males, the resulting heterozygous *tsl*/+ offspring are more temperature sensitive than the wild type strain, but less sensitive than the homozygous *tsl* strain. This finding, in combination with the result that the heterozygotes generated by the reciprocal cross are not sensitive, points towards an incomplete maternal effect of this *tsl* mutation (G. Franz, unpublished results). Furthermore, the sensitivity seems to be independent of the dose, i.e. *tsl*/+/+ genotypes are as sensitive as *tsl*/*tsl*/+ and *tsl*/+ genotypes [3].

Although the egg stage is the most temperature sensitive phase of the life cycle, the other stages, i.e. larvae, pupae and adults, also show varying degrees of sensitivity (G. Franz, unpublished results). On the one hand, this makes it necessary to avoid elevated temperatures during mass rearing, though only for that fraction of the production that is used to set up the next generation of the colony. Here the temperature is quite important, primarily because it affects indirectly the stability of the sexing system. Removal of the bacterial and fungal contamination of the diet by various means, and the use of liquid diets (P. Rendon, personal communication) in combination with the appropriate diet tray design (J. Hendrichs, personal communication) are possible alternatives for minimizing the temperature related effects. On the other hand, the temperature sensitivity of later stages, especially of *tsl* female adults, can be viewed as an additional safety feature, i.e. accidental release or escape from the factory will not be a problem as these flies will not survive.

2.2. Y–autosome translocations

The wild type allele of the respective selectable marker is linked to the male sex via a Y–autosome translocation [4]. In the medfly, the Y chromosome contains a dominant male determination factor [5]. The males in a sexing strain are heterozygous for the selectable marker, i.e. the free autosome carries the mutant allele. The following three primary deviations from pseudolinkage were observed during the analysis of various sexing strains.

2.2.1. Recombination in heterozygous males

During the mass rearing of genetic sexing strains it became apparent that sexing systems can break down over time. It was determined that genetic recombination in the males is the primary cause of this instability [6]. Genetic exchange in the chromosomal region between the translocation breakpoint and the location of the selectable marker will unlink the wild type allele from the male sex. This leads in the following generation to mutant males and wild type females. Especially the latter type of aberrant fly poses a severe problem because these females cannot be separated from the males and are therefore released. The recombination frequency in males is

very low [7]. However, as the resulting recombinant females are wild type, they possess a selective advantage over their mutant female siblings. This results in an accumulation rate that exceeds the underlying recombination rate. In the case of the *tsl* based sexing system, the recombinant females that have lost the *tsl* would accumulate faster if the rearing temperature were not maintained properly or if the next generation were set up exclusively with early pupae.

One strategy to overcome this stability problem is to generate translocations where the Y–autosome breakpoint is close to the selectable marker. This required two separate lines of experiments. First, the chromosomal location of the selectable marker had to be determined. Two mutations, *wp* and *tsl*, were mapped on the right arm of chromosome 5 [3, 8]. Secondly, a series of translocations, linking chromosome 5 to the Y chromosome, had to be induced and analysed with respect to the position of the Y–autosome breakpoint [8–10]. It was shown that sexing strains based on translocations with breakpoints close to *wp* and *tsl* are sufficiently stable that they can be reared for many generations without stability problems [9].

A second strategy to avoid the occurrence of recombinant adults is to incorporate pericentric inversions. In genotypes heterozygous for such an inversion, recombination in the inverted chromosome segment will lead to unbalanced gametes and, consequently, the resulting offspring will not be viable. We have initiated experiments to induce homozygous viable pericentric inversions in the *wp-tsl* chromosome. Applied to genetic sexing, such a strain would consist of females homozygous for the inversion (i.e. although female recombination frequency is high, this does not lead to semisterility) and males containing (a) a Y–autosome translocation with a breakpoint close to *wp* and *tsl*, and (b) a free autosome with the pericentric inversion. Male recombination between the breakpoint and the selectable markers is low and, consequently, the resulting sterility should be negligible, especially compared with the sterility caused by meiotic segregation of Y–autosome translocations.

Very little is known about recombination between the sex chromosomes in the medfly. Potentially, even unequal recombination between the two Y fragments of a Y–autosome translocation (or intrachromosomal recombination of the Y) is possible, especially considering the highly repetitive structure of the Y chromosome. The structure of the Y chromosome in certain aberrant flies, isolated from a mass rearing colony, might be evidence for this (U. Willhoeft, personal communication).

2.2.2. *Adjacent-1 segregation*

The segregation of Y–autosome translocation during the male meiosis is complex. Only alternate segregation produces balanced gametes, while adjacent-1 segregation generates genotypes with Y chromosome deletions and either autosome deletions or duplications. The frequency of such genotypes at any developmental stage depends on the following factors.

(a) *Relative ratio of alternate to adjacent-1 segregation*

It is very difficult to measure this ratio directly. However, by comparing egg hatch, pupation and emergence with a wild type strain one can deduce that some translocations, e.g. T(Y;5)30C, show primarily alternate segregation, while in the case of other translocations both types occur at equal frequencies. It is possible that the position of the breakpoint on the Y chromosome, relative to the Y centromere, is responsible for these differences.

(b) *Viability of these unbalanced karyotypes*

It is assumed that in most cases autosomal deletion leads to early, i.e. zygotic, lethality. A very short deletion, e.g. segment 61A to 62D (trichogen map), can potentially survive until the larval stage as judged by the egg hatch of more than 80% in the case of translocation T(Y;5)3-245 [10] (G. Franz, unpublished data). The viability of triplications appears to be inversely correlated to the length of the duplicated segment [10]. In many translocation strains, a certain fraction (up to 20% of all emerged adults) of the adjacent-1 segregation offspring (general structure: X/Y-A/A/A) reaches adulthood and can be made visible by the appropriate combination of mutations [3, 11]. The sex of these flies is dependent on the position of the Y-chromosomal breakpoint relative to the male determination factor (U. Willhoeft et al., in preparation). Generally, adjacent-1 flies are weak: their overall fitness and viability are reduced significantly. Furthermore, they are more temperature sensitive than balanced genotypes (G. Franz, unpublished results).

The sterility of Y–autosome translocation bearing males is directly proportional to the complexity of the translocation [8]. The more autosomes are involved, the more complex is segregation and the more unbalanced genotypes are generated. Therefore, for mass rearing only simple translocations are considered.

2.2.3. Non-disjunction

Non-disjunction of X and Y chromosomes has been reported [5]. Preliminary data from the Agency's Laboratories in Seibersdorf, near Vienna, show that certain Y–autosome translocations produce at relatively high frequencies unbalanced genotypes with two X chromosomes and one of the two translocation fragments, usually the one with the Y centromere. As this is not the case for all strains, one might speculate that the position of the Y-chromosomal breakpoint, and the resulting difference in size/quality of the two Y fragments, are responsible for this phenomenon. Whether these aberrant genotypes pose a threat to the sexing system depends on the position of the autosomal breakpoint (which determines the phenotype with respect to the selectable marker) in combination with the position of the Y-chromosomal

breakpoint (which determines whether the aberrant fly is male or female). A serious problem for mass rearing arises when wild type females are generated, while mutant males can be tolerated as long as their frequency does not severely reduce recovery.

2.3. Mass rearing of sexing strains based on *tsl* mutation

Several translocations have been used to construct sexing strains utilizing the temperature sensitive lethal as selectable marker, namely T(Y;3;5)1-56 (= GS-2), T(Y;2;5)2-82 (= GS-3), T(Y;5)1-61 (= GS-4), T(Y;5)2-22 (= GS-6) [3, 9] and T(Y;5)3-245 (= GS-8) [10]. These were selected on the basis that they have Y–autosome breakpoints on the right arm of chromosome 5 and show very good stability in laboratory tests. The strains GS-2 and GS-3 were used only for a relatively short period of time as they are difficult to rear owing to the complexity of the translocation and the resulting sterility of the males. The most promising results were obtained with GS-4, later named VIENNA-42.

The homozygous *tsl* strain and all translocation strains were outcrossed with our wild type strain EgII before they were combined to generate sexing strains and before they were transferred to our mass rearing facility. This was done (a) to remove other mutations present in the *tsl* strain after EMS mutagenesis and (b) to increase genetic variability. The outcrossing scheme was the same as the one proposed later for the outcrossing with genetic material from Guatemala. However, here no complications with sterility were encountered. This is most likely due to the fact that all our strains, including the translocations, are based on the EgII strain.

The mass rearing procedure had to be modified compared with the scheme used for rearing a sexing strain utilizing only *wp* as selectable marker:

- — The adult colony was enlarged to obtain sufficient numbers of eggs.
- — That fraction of the production that was put aside for setting up the next generation was reared separately.
- — Especially for this fraction, temperature was controlled more carefully throughout the rearing process.
- — Owing to the presence of the *tsl* mutation, the females pupate, on average, later than the males. This feature was used to set up the next generation primarily with material from pupal collections 3 to 5, thereby selecting against recombinant females that have lost the *tsl* and therefore pupate early, together with the males.
- — To obtain only males, eggs were collected for 24 h and then treated with temperature during the 'bubbling' phase.

With these modifications it is possible to mass rear VIENNA-42 and to produce only males. On two occasions this strain was reared either for 21 or for 13 generations;

in both cases the level of recombinant females stayed below 1% (J. Hendrichs, unpublished results). However, increasing numbers of males emerging from white pupae were observed. Preliminary genetic tests show that these males have apparently lost the translocation (G. Franz, unpublished results). This was confirmed by cytological analysis on a few individuals (U. Willhoeft, personal communication). It is not clear yet whether these aberrant males are the consequence of intra-Y-chromosomal recombination or whether they were introduced into the strain during the outcrossing with EgII. As this phenomenon was not observed when GS-6 was mass reared for eight generations, we have started to investigate the potential of this strain.

2.4. Construction of VIENNA-42/Guatemala hybrid strain

In preparation for a large scale mass rearing and field test of the medfly sexing strain VIENNA-42 in Guatemala, it was suggested that the genetic background of this strain (derived from a wild type strain originating from Egypt) be replaced with genetic material from Guatemala. The following three arguments were considered:

— To increase the genetic variability
— To improve mating competitiveness relative to the target population
— To avoid introduction of non-native flies.

The two components of the sexing strain VIENNA-42, the temperature sensitive lethal (*tsl*; linked to the mutation *wp*) and the Y–autosome translocation T(Y;5)1-61, were outcrossed separately.

The original outcrossing scheme for the *tsl* consisted of three consecutive crosses with flies from a recently colonized mass rearing strain from Guatemala (Toliman strain). In the last two crosses, Toliman females were used, thereby maintaining the *wp-tsl* chromosome in the males to avoid recombination between these markers. In the next step, an attempt was made to reconstruct a strain homozygous for *wp* and *tsl*, i.e. the F_4 was inbred, followed by single-pair crosses with *wp* individuals. However, it became apparent that these females were sterile, i.e. they produced no or only very few eggs and all dissected females showed various forms of degenerated ovaries. This phenomenon gradually disappeared when the *wp-tsl* chromosome was maintained for three or more generations in a heterozygous condition. From these generations it was possible to recover 27 viable *wp* families and 9 of these contained the *tsl* mutation. By crossing females of 8 of these families with T(Y;5)1-61 males (also outcrossed with Guatemalan material), the VIENNA-42/Guatemala hybrid strain was constructed.

At the stage of outcrossing where all inbred females were sterile, alternative strategies were considered to overcome this problem. As the observed sterility was

reminiscent of a phenomenon called hybrid dysgenesis, we started a second set of crosses:

(a) The reciprocal outcrossing scheme: i.e. only the males were used from the Guatemala strain and the *wp-tsl* chromosome was maintained in the females.
(b) A second *tsl* subline was used.
(c) A second strain from Guatemala was used.

In all crosses severe sterility was observed. However, in general, using the males from either of the two Guatemala strains generated less severe effects. However, the recovery of *wp-tsl* families was reduced. This is due to the fact that the labelled chromosome had to be maintained in females in a heterozygous condition, resulting in an increased recombination between the two mutations.

2.5. Evaluation of VIENNA-42 under field cage and field conditions

So far, three extensive evaluations of the behaviour of VIENNA-42 have been performed:

(a) Tests on potted host and non-host trees in the greenhouse at Seibersdorf, assessing all aspects of the behaviour of these males in comparison with males of other laboratory strains used previously with success in the field;
(b) Tests involving the release of sterile VIENNA-42 males from a central point in a citrus area of Chios, Greece, to study their dispersal and survival under field conditions;
(c) Tests on field caged orange trees in Chios to assess the competitiveness of VIENNA-42 males relative to wild males for wild females.

Results of the first tests indicate that VIENNA-42 males exhibit all components of the normal sexual behaviour of this species, as well as a pattern of temporal and spatial distribution of activities similar to other strains [12]. The field dispersal and survival study showed mortality rates of sterile VIENNA-42 males comparable with results of survival studies of standard strains, with dispersal exceeding 100 m, the minimum distance required between 200 m flight lines during conventional sterile fly releases [12].

The field cage study indicated that sterile VIENNA-42 males attract a greater number of receptive females than wild males. However, wild males still had a moderate competitive advantage (confirmed by egg sterility) in terms of mating success, indicating some rejection of VIENNA-42 males by wild females during final courtship. It is concluded that VIENNA-42 is a viable genetic sexing strain, with a competitiveness comparable with that of other standard medfly laboratory strains.

3. CONCLUSIONS

Genetic sexing systems have reached a sufficient level of accuracy, stability and applicability that large scale mass rearing of these strains is possible. On the basis of experience that will be gained in this process, further refinements may be required in the future, e.g. development of optimized rearing with respect to temperature treatment and temperature control during the larval stages. Furthermore, additional measures to further improve stability could be included in the sexing strains. However, the existing systems have two principal drawbacks:

(a) As these strains are based on Y–autosome translocations, their inherent semisterility requires increased egg production.
(b) The transfer to other pest species is very difficult, i.e. for each species for which genetic sexing systems have to be developed, a rather extensive basic knowledge in genetics and cytology has to be established.

It is hoped that these limitations can be overcome by generating sexing systems with molecular techniques. Several laboratories are working on the identification of genetic elements that could be used as vectors in a transformation system for pest insects, and other groups have initiated research to clone genes that, after appropriate engineering and reintroduction into the insect, can be used as selectable markers in genetic sexing systems.

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