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Development of Genetic Sexing Strains in Lepidoptera: from Traditional to Transgenic Approaches

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ABSTRACT The sterile insect technique (SIT) is currently being used for the control of many agricultural pests, including some lepidopteran species. The SIT relies on the rearing and release of large numbers of genetically sterile insects into a wild population. The holokinetic chromosomes of Lepidoptera respond differently to radiation than do species where there is a localized centromere. This difference has enabled a variation of the SIT to be developed for Lepidoptera where a substerilizing dose of radiation is given to the insects before their release with the result that a certain level of sterility is inherited by the F_1 offspring. The development of genetic sexing strains for fruit flies, enabling the release of males only, has resulted in enormous economic benefits in the mass rearing and has increased the efficiency of the field operations severalfold. This article outlines Mendelian approaches that are currently available to separate large numbers of males and females efficiently for different lepidopteran species and describes their difficulties and constraints. Successful transgenesis in several lepidopteran species opens up new possibilities to develop genetic sexing strains. The proposal to develop genetic sexing strains described in this article takes advantage of the fact that in Lepidoptera, the female is the heterogametic sex, with most species having a WZ sex chromosome pair, whereas the males are ZZ. This means that if a conditional lethal gene can be inserted into the W chromosome, then all females should die after the application of the restrictive condition. The assumptions made to accommodate this model are discussed, and the advantages to be gained for control programs are elucidated.

KEY WORDS Lepidoptera, codling moth, sterile insect technique, genetic sexing, insect transgenesis

THE STERILE INSECT TECHNIQUE (SIT) is now an established component of many integrated approaches to insect pest control (Tan 2000). It relies on the mass rearing and release of large numbers of genetically sterile insects into a wild population. Matings between the released sterile males and the wild females produce no progeny. Repeated release of the sterile insects results in effective control and under certain ecological situations, elimination of local populations. Genetic sterility in the released insects is induced by ionizing radiation (Robinson 2002a). The paradigm of this method of pest control has been the eradication of the New World screwworm, *Cochliomyia hominivorax* (Coquerel), from the southern United States, Mexico, and Central America (Krafsur 1998, Wyss 2000).

Building on the success of this program the technique was developed for many agricultural pests, including several lepidopteran species (LaChance 1985; Dyck and Gardiner 1992, Henneberry 1994). In comparison with dipteran species, it soon became clear that Lepidoptera require much higher levels of ionizing radiation to obtain full sterility (LaChance 1967, Tazima 1978, LaChance and Graham 1984). Molecular mechanisms for the high radioresistance in Lepidoptera might include an inducible cell recovery system and/or a DNA repair process (Koval 1996). Nevertheless, the main cause of this difference is thought to reside in the different kinetic organization of chromosomes in these two groups of insects. The Diptera possess typical monocentric chromosomes with ki-

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netic activity restricted to the centromere, whereas lepidopteran chromosomes are essentially holokinetic (Gassner and Klemetson 1974; Murakami and Imai 1974; reviewed by Wolf 1994, 1996). They lack distinct primary constrictions (the centromeres), and their kinetic activity is distributed along most of the chromosome length. The holokinetic (or holocentric) chromosome structure ensures that most radiation-induced breaks do not lead to the loss of chromosome fragments as is typical in species with monocentric chromosomes. It also reduces the risk of lethality caused by the formation of unstable aberrations such as dicentric chromosomes (Marec et al. 2001, Tothová and Marec 2001).

The impact of chromosome structure on the biological consequences of radiation-induced lesions and the behavior of chromosomal aberrations have enabled a variation of SIT to be developed for Lepidoptera. The genetic damage produced by substerilizing doses of radiation is inherited, and in some cases the F_1 individuals are completely sterile. This phenomenon has been called inherited sterility (IS) and has been extensively studied in pest species, both in terms of its underlying scientific basis (North and Holt 1970, Anisimov et al. 1989, Tothová and Marec 2001) and its application (Carpenter and Gross 1993; Bloem et al. 2001; reviewed by North 1975, Carpenter et al. 2005).

Models predict that the release of substerile males only is the most efficient approach to population suppression of lepidopterous pests compared with the release of only fully sterile males or indeed bisexual releases (Anisimov and Shvedov 1996). The development of a genetic sexing strain and the subsequent release of male moths only, seem to have many benefits, e.g., they would 1) reduce assortative mating; 2) allow for a lower dose of radiation to be used on males, thereby increasing their competitiveness (Carpenter et al. 1989); 3) reduce rearing costs; 4) decrease the concern that growers and industry representatives may have regarding the release of females causing damage to the fruits, even if they are sterile; and 5) for species such as codling moth, *Cydia pomonella* (L.), where there is a facultative diapause (Garcia-Salazar et al. 1988, Howell and Neven 2000), the ability to stockpile only male pupae would have additional significant economic advantages (Bloem et al. 1997).

In SIT programs against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Robinson 2002b), the benefits of releasing males only in terms of strain performance, only became apparent when large field evaluations were conducted (Rendon et al. 2004), and to date, the results from field-cage tests remain inconclusive. In Guatemala, the release of males only resulted in a five-fold increase in the rate of induced sterility in the native fly population compared with bisexual releases (Rendon et al. 2004). Therefore, large-scale field evaluations also will be needed to quantify the efficiency of male only releases versus bisexual releases in Lepidoptera SIT or IS programs.

This article outlines the approaches that are currently available to separate efficiently large numbers of males and females for different lepidopteran species and describes the difficulties and constraints of these Mendelian approaches. Transgenesis can now be carried out in several lepidopteran species, and this opens up additional possibilities to develop genetic sexing strains. We propose a new approach for the development of genetic sexing strains in Lepidoptera, which is based on the construction of transgenic females carrying a dominant conditional lethal gene in the female-determining chromosome, the W. The strength of the proposed approach is the introduction of a number of internal safeguards to avoid the release of transgenic insects.

Sex Chromosomes and Sex Determination in Lepidoptera

Knowledge of the sex chromosome system is essential for the development of genetic sexing strains in any target pest species. In Lepidoptera, the chromosome mechanism of sex determination is of the WZ type: females are heterogametic, males homogametic. Among the relatively few species with identified sex chromosomes the majority have a WZ/ZZ (female/male) system or its numerical variations, such as Z/ZZ, W_1W_2Z/ZZ, and $WZ_1Z_2/Z_1Z_1Z_2Z_2$, where subscript numbers indicate multiple sex chromosomes (Suomalainen 1969, Robinson 1971, Nilsson et al. 1988, Traut and Marec 1997, Rishi et al. 1999).

Basic characteristics of W and Z chromosomes are similar to those of XY systems. Like many Y chromosomes, the lepidopteran W chromosomes consist partly to wholly of heterochromatin, whereas the Z chromosomes consist of transcriptionally active euchromatin. Accordingly, almost no genes are known on W chromosomes, whereas Z chromosomes are rich in genes (Traut 1999; Koike et al. 2003). Moreover, the W chromosomes are responsible for a peculiarity of the lepidopteran genome: like mammals, Lepidoptera possess female-specific sex chromatin. Most species display one or more heterochromatin bodies in female somatic interphase cells but not in male cells. This female specific so-called "W-chromatin" or "sex-chromatin" is derived from the W chromosome. Because sex chromatin is easily discernible in interphase nuclei and especially so in the highly polyploid somatic cells, it is a useful marker for diagnosing the chromosomal sex of embryos and larvae and for identifying sex chromosome aberrations in mutagenesis screens (Traut and Marec 1996).

The females of Lepidoptera lack crossing over, and their meiosis is achiasmatic (Nokkala 1987, Marec 1996). Nevertheless, the W and Z sex chromosomes, although often largely nonhomologous and of different sizes, pair completely during meiotic prophase I and form a regular synaptonemal complex. In a number of lepidopterans, the WZ bivalent can be easily identified in pachytene oocytes by the heterochromatic thread of the W chromosome (Marec and Traut 1994, Traut and Marec 1997). Moreover, advanced methods of molecular cytogenetics have recently become available for sex chromosome identi-

fication. Traut et al. (1999) modified comparative genomic hybridization for the study of molecular differentiation of sex chromosomes in both the XY and WZ systems. This method makes the identification of the Y or W possible even in species with homomorphic sex chromosomes if they differ sufficiently in their gross DNA composition. Alternatively, the W chromosome can be identified using genomic in situ hybridization of female-derived genomic probes (Sahara et al. 2003b, Mediouni et al. 2004). Furthermore, Sahara et al. (2003b) demonstrated the identification of the W chromosome in the silkworm, *Bombyx mori* (L.), by using fluorescence in situ hybridization with W chromosome-derived bacterial artificial chromosome probes.

Unfortunately, little is known about the actual genetic control of sex determination in Lepidoptera, and primary sex-determining factors remain to be discovered. Available evidence suggests that the mechanisms are diverse. Data from inheritance of irradiated chromosome fragments suggest that the sex is strongly controlled by the presence or absence of a specific region of the W chromosome in *B. mori* (Tazima 1964). This led to the prediction long ago that the W chromosome carries an epistatic feminizing gene that determines female development. So far, however, none has been found in *B. mori* or other lepidopterans. By contrast, results obtained in sex chromosome mutants of the Mediterranean flour moth, *Ephestia kuehniella* Zeller, suggest that the W chromosome carries a male killing factor that might serve as a feedback control of sex rather than as a determinant in the female sex differentiation pathway (Marec et al. 2001). However, the absence of a W chromosome in "primitive" Lepidoptera and frequent secondary loss of the W in "advanced" Lepidoptera favor balance mechanisms of sex determination with Z-linked male promoting factors instead of W-linked female promoting factors (Traut and Marec 1996).

Ohbayashi et al. (2001) isolated from *B. mori* a homologue of the *doublesex* (*dsx*) gene that is a downstream member of the sex-determining cascade in *Drosophila melanogaster* (Meigen). *B. mori doublesex* (*Bmdsx*) is present on autosomes in both sexes and, like *Drosophila*, is alternatively spliced to yield male- and female-specific mRNAs (Suzuki et al. 2001). Recently, Suzuki et al. (2003) showed that *Bmdsx* encodes male-specific (BmDSXM) and female-specific (BmDSXF) polypeptides and that the BmDSXF protein has a regulatory function in females that acts repressively in males. The results strongly support the view that *Bmdsx* acts early in the hierarchy of regulatory genes controlling female differentiation in *B. mori.* However, sex-specific splicing is regulated by repression of the default female-specific processing pattern (Ohbayashi et al. 2002) and thus very different from that in *Drosophila*, where the default state is male-specific splicing, and female-specific splicing is under control of positive splicing cofactors (Graham et al. 2003).

Genetic Sexing Strains Developed in Lepidoptera

Approaches based on selectable genes and translocations used for the development of genetic sexing strains in *C. capitata* (Robinson 2002b) also have been used in *B. mori*, e.g., sex-linked visible traits such as egg color, cocoon color, or a larval phenotype (Tazima 1964, Nagaraju 1996). However, the most useful type of sexing mechanism developed for Lepidoptera has been based on the construction of balanced lethal (BL) strains essentially following the scheme initially proposed by Strunnikov (1975) for the silkworm. In sericulture facilities, males produce ≈20% more silk than females, and the mass rearing of only male progeny brings therefore significant economical benefit (Strunnikov 1987). To date, BL strains have been developed in two species: *B. mori* (Strunnikov 1975, 1987; Ohnuma and Tazima 1983; Ohnuma 1988) and *E. kuehniella* (Marec 1991, Marec et al. 1999). In an optimum design, such a BL strain consists of males that are *trans*-heterozygous for two nonallelic, sex-linked recessive lethal mutations (SLRLMs). The two lethal loci (e.g., *sl-1* and *sl-2*) should be located close to one another to minimize the probability of recombination between them. Females carry either *sl-1* or *sl-2* on their Z chromosome as well as a portion of the Z chromosome translocated onto the W chromosome. The T(W;Z) translocation includes wild-type alleles of both lethal loci, protecting the females from the expression of either lethal allele (Fig. 1). Half of the male progeny are eliminated during development (preferably during embryogenesis), because one of the SLRLMs is present in a homozygous state. The surviving males are those balanced for two SLRLMs. For sexing, BL males are mated to females of a wild-type strain, yielding progeny consisting exclusively of males. All female progeny die because they are hemizygous for either of the SLRLMs (Fig. 1). A critical difference of these strains from those developed in Mediterranean fruit fly is that BL males have to be selected and mated to wild females, whereas in *C. capitata*, the sexing properties are within a single strain and specific crosses are therefore not required.

Balanced lethal strains can be developed in any lepidopteran species except those lacking the heterologous W chromosome in the female sex. Three steps are required to construct such a strain: 1) isolation of SLRLMs induced in males by a mutagen (e.g., ethyl methanesulfonate) causing a point mutation or small deletion (Marec 1990), 2) isolation of T(W;Z) translocations induced in females by ionizing radiation (e.g., gamma-rays) (Marec and Mirchi 1990), and 3) construction of the BL strain by using a multistep breeding scheme (Marec 1991). For each step, it is advisable to use a genetic marker. In *E. kuehniella*, for example, the use of the sex-linked recessive mutation *dz* (dark central field of forewings; Marec 1990) facilitated the isolation and rearing of SLRLM and T(W;Z) mutant lines as well as the construction of a BL strain (Marec 1991). Three good Z-linked marker genes could be used in a similar manner in the silkworm (Fujii et al. 1998). These are *os* (larval skin

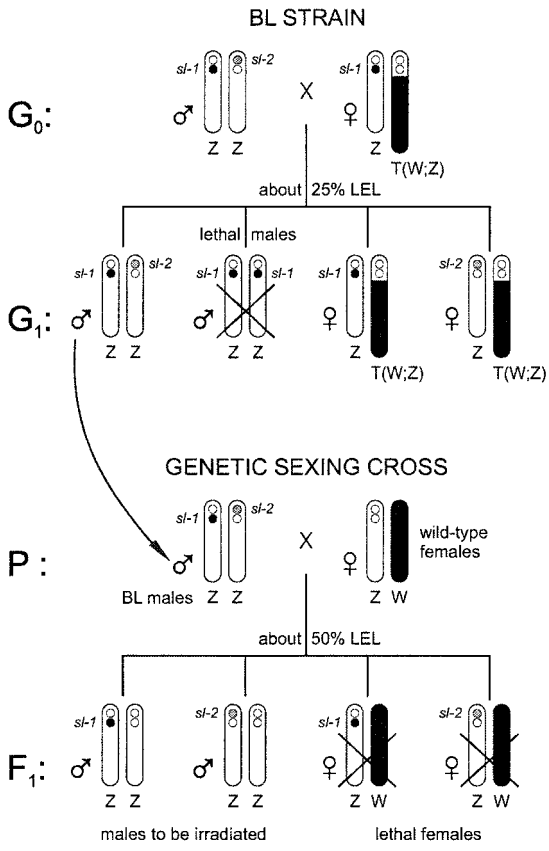

Fig. 1. Scheme of genetic sexing system for Lepidoptera based on the construction of balanced lethal males (Strunnikov 1975). BL, balanced lethal strain; Z and W, sex chromosomes; *sl-1* and sl-2, sex-linked recessive lethal mutations; LEL, late embryonic lethality; T(W;Z), W chromosome carrying translocated Z-chromosome segment. Genetic sexing: crosses between BL males and wild-type females produce all-male progeny that can be irradiated and released.

translucent), *sch* (chocolate body color of newly hatched larvae), and *e* (larval body shape elongated). Unfortunately, similar marker mutations are not available in most key lepidopteran pests.

In the silkworm, Strunnikov and coworkers constructed a slightly modified BL strain with the help of the recessive, Z-linked marker *os* (reviewed in Strunnikov 1987). Because they were not successful in obtaining the optimal SLRLM loci, they used only one SLRLM, in which the expression in females was rescued by the wild-type allele of the T(W;Z) translocation, whereas the other one was not rescued, but was located close to the first one. In this strain, 50% of the progeny died: one-half belonging to the males homozygous for the first SLRLM and one half being the females hemizygous for the other SLRLM. Progeny of crosses between BL males and normal females of commercial strains consisted of 99.59% males under experimental conditions and up to 99.85% males under conditions of silkworm breeding facilities.

Two different BL silkworm strains were developed in Japan, of which the first had a rather complicated genetic structure (Ohnuma and Tazima 1983; Ohnuma 1988). Males were *trans*-heterozygous for two autosomal recessive lethal mutations, located on chromosome 5. Females carried a T(W;5) translocation that involved wild-type alleles of both lethal loci and a single chromosome 5 with one of the lethal mutations. Only male progeny were produced by mating BL males to mutant females which carried a similar T(W;5) translocation but without wild-type alleles of the lethal loci and a single chromosome 5 without lethal mutations. Later, Ohnuma (1988) successfully constructed another BL strain exactly according to Strunnikov's scheme, that is, consisting of males balanced for two Z-linked SLRLMs and of females carrying a T(W;Z) translocation.

The flour moth is currently the only lepidopteran pest in which genetic sexing is possible by using Strunnikov's scheme. In the BL-2 strain constructed (Marec 1991), males are balanced for two nonallelic SLRLMs, *sl-2* and *sl-15*. The lethal loci exhibit tight linkage (0.5% recombination). Females carry the T(W;Z)2 translocation which involves wild-type alleles of both lethal loci. When BL-2 males were mated to wild-type females, the progeny consisted of 99.74% males, and a few females that represented recombinants (0.26%). Laboratory experiments confirmed the potential that BL-2 males can be used for genetic sexing, particularly in combination with population suppression by the release of partially sterile males (Marec et al. 1999). In these experiments, BL-2 males were outcrossed to wild-type females one generation before irradiation. The outcrosses produced only male progeny that were heterozygous for either *sl-2* or *sl-15* (*sl*/+ males). The male progeny were irradiated and induced inherited sterility was measured by mating to wild-type females. The experiments revealed two additional benefits of the use of BL males for genetic sexing: 1) the outcrosses one generation before irradiation improve competitiveness of *sl*/+ males to be released through heterosis, and 2) *sl*/+ males introduce not only radiation-induced, deleterious genetic changes into native populations but also SLRLMs that reduce the number of females and thus contribute to population suppression.

Unfortunately, the use of BL strains for genetic sexing is not easily applicable under mass rearing conditions. In particular, the necessity to maintain two strains (BL and wild type) in the rearing facility without contamination, as well as the need to sex each of these strains, represent major drawbacks of this sexing technique. Other significant obstacles must be overcome before this genetic sexing system can be used for suppression of key pests such as the codling moth. For example, suitable markers are currently lacking for the construction of BL strains in this species. Also, the constructed mutant BL strain must be routinely checked to prevent the loss of its genetic structure through genetic recombination or colony contamination (Marec et al. 1999).

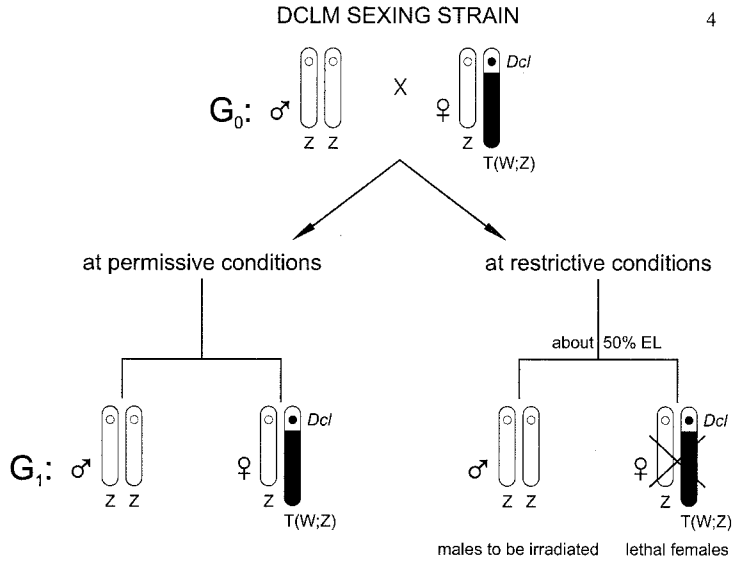

Fig. 2. Scheme of genetic sexing strain based on a dominant conditional lethal mutation (DCLM, *Dcl*), translocated from the Z chromosome onto the W chromosome; EL, embryonic lethality; T(W;Z), W chromosome carrying translocated Z-chromosome segment. Genetic sexing: the DCML strain, if kept at restrictive conditions, produces exclusively male progeny that can be irradiated and released.

Use of Dominant Conditional Lethal Mutations for Obtaining Solely Males

The use of a genetic sexing system that is based on a dominant conditional lethal mutation (DCLM), linked to the female-specific W chromosome, could solve the problems of the propagation of the two strains. However, heterochromatic W chromosomes of most Lepidoptera probably contain very few or no genes (Traut 1999, Traut et al. 1999, Sahara et al. 2003a), in which such DCLM could be induced by standard mutagenesis. Besides the proposed, but not yet identified feminizing gene (Ohbayashi et al. 2002), only three W-linked genes have so far been reported. These are the egg size determining gene, *Esd*, in *B. mori* (Kawamura 1988), a homologue of the Z-linked *period* gene in the silkmoth *Antheraea pernyi* (Guérin-Méneville) (Gotter et al. 1999), and possibly a gene responsible for the forewing color in the tiger swallowtail butterfly, *Papilio glaucus* L. (Scriber and Evans 1987, Andolfatto et al. 2003). Therefore, it would certainly be easier to induce a DCLM in another chromosome, preferably in the gene-rich Z chromosome, and then translocate it onto the W chromosome by irradiation of females (Fig. 2). Such a DCLM would permit production of both sexes when kept at permissive conditions, whereas solely male progeny would be obtained at restrictive conditions. This approach is theoretically simple and ensures complete elimination of females but has a considerable weakness. In spite of their potential for insect population suppression (Klassen et al. 1970), no DCLMs have been isolated from any lepidopteran species so far. It is obvious that any attempt to construct a sexing strain based on DCLMs would require a labor-intensive systematic study to obtain a desirable mutation in a particular pest.

Transgenic Approach to Genetic Sexing in Lepidoptera

The development of genetic sexing strains based on W-linked DCLMs that kill all females while allowing release of only mass-reared radiation-sterilized males can be accomplished through the use of transgenesis. Successful and stable germline transformation has been achieved in several lepidopteran species by using the transposable element *piggyBac* (Fraser 2000) with the marker gene enhanced green fluorescent protein (EGFP) from the jelly fish *Aequorea aequorea* (Forskal), under the *Actin A3* promoter from *B. mori*. These include the silkworm, *B. mori* (Tamura et al. 2000, Uhlirova et al. 2002), the pink bollworm, *Pectinophora gossypiella* (Saunders) (Peloquin and Miller 2000, Peloquin et al. 2000), and the codling moth (Ferguson et al. 2004). In the latter species, transgenic strains have been in production since 1995 and have been propagated through 30 generations (Ferguson et al. 2004). From this research, it is apparent that transgenesis in lepidopteran pests is feasible (Wimmer 2003), however, in comparison with transformation in dipterans it is certainly not yet routine. Efficiencies of transgenics are lower in lepidopterans, but improving with better vectors and injection techniques, and that establishing stable transgenic lines is somewhat more labor intensive and takes longer because of longer life cycle.

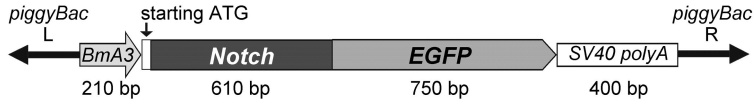

Fig. 3. Design of the *piggyBac* vector used for genetic transformation of the codling moth; a mutant allele of the *Notch* gene, N^{60g11}, in tandem with the *EGFP* marker gene is located behind the *B. mori Actin A3* promoter (*BmA3*). *piggyBac* L and *piggyBac* R, left and right *piggyBac* interrupted sequences; starting ATG, a codon starting translation of the fused Notch/EGFP protein; *SV40 polyA*, simian virus 40 polyadenylation site to stabilize transcription. Size of inserted DNA sequences is given in base pairs (bp) below each insert.

To generate a genetic sexing strain in a lepidopteran pest, it is desirable to insert into the W chromosome a DCLM of a known gene that is conserved in insects, essential for ontogenic development, and preferably expressed during embryogenesis. One of the potential candidates with these characteristics is the *Notch* gene, initially described in *D. melanogaster*. *Notch* is a complex gene that plays a major role in intercellular communication and cell fate control (Welshons and von Halle 1962). The Notch signaling pathway is widely conserved in animals and is involved in a variety of biological processes such as cell differentiation, cell polarity, and proliferation (reviewed by Campos-Ortega and Knust 1990, López-Schier 2003). One of the *Notch* mutant alleles, N^{60g11}, has been proposed for autocidal biological control of insect pests through genetic transformation. The N^{60g11} allele encodes a truncated form of the Notch protein that is temperature sensitive and was shown to result in the death of heterozygous *Drosophila* embryos kept <20°C (Fryxell and Miller 1995). Thus, N^{60g11} causes dominant, cold-sensitive lethality and seems to be well suited for genetic sexing. In addition, a plasmid construct containing the *piggyBac* transposon with N^{60g11} in tandem with the *EGFP* marker gene under the *Actin A3* promoter is available (Fig. 3).

A theoretical scheme for development of the proposed genetic sexing strain through the use of germline transgenesis is given in Fig. 4. In this strain, females would carry in the W chromosome an inserted construct consisting of a functional vector (the *piggyBac* transposon), a DCLM of the target gene (e.g., N^{60g11}), and a reporter/marker gene (e.g., *EGFP*) driven by a ubiquitous nonconditional promoter (e.g., *Actin A3*). Under permissive conditions (e.g., room temperature for N^{60g11}) the progeny would consist of wild-type males and transgenic females, because the W chromosome with the transgene would be inherited only via the female sex. In egg collections treated with restrictive conditions (e.g., 15°C for N^{60g11}), all female progeny would be eliminated during embryogenesis by the presence of the DCLM transgene on the W chromosome. Surviving male embryos would be transferred to permissive conditions for hatching, reared to the late pupal stage or adulthood, irradiated, and released.

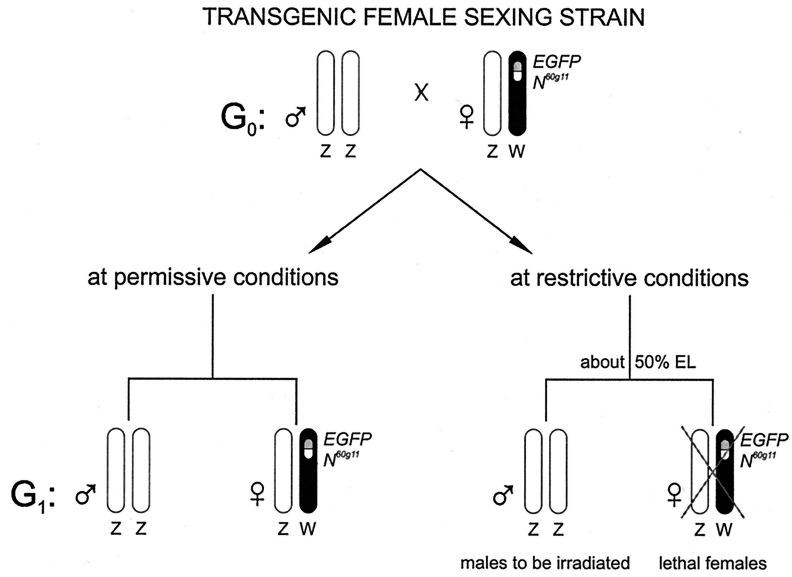

Fig. 4. Scheme of genetic sexing strain based on the use of transgenic females that possess an insert in their W chromosome, containing the *EGFP* marker gene and the mutant allele of the *Notch* gene (N^{60g11}). EL, embryonic lethality. Genetic sexing: the transgenic female strain, if kept at restrictive conditions, produces exclusively male progeny that can be irradiated and released.

Strengths and Weaknesses of the Transgenic Approach

The transgenic approach may represent a straightforward method for generating genetic sexing strains in lepidopterans, and has several beneficial features. The main advantage of such a transformed sexing strain is that only the female progeny would contain the transgene, leaving the males with a fully wild-type genome. Thus, competitiveness of released males would not be negatively affected by pleiotropic effects of the DCLM gene used for elimination of females. In addition, because the released males would not contain the transgene, the release program would be less likely to be rejected publicly on the basis of concerns about genetically modified organisms. Also if some females were to survive, they would have received a sterilizing dose of radiation (>100 Gy) before release, thereby being unable to produce viable eggs and even if nonirradiated females accidentally escaped from the colony, then they would be unable to propagate the transgene under temperatures <20°C, which are common in a temperate climate.

The transgenic approach will facilitate mass rearing as well as the implementation of SIT programs. For example, monitoring of embryonic lethality can be accomplished easily by screening egg sheets for the expression of a fluorescent marker protein after the treatment at restrictive conditions. Monitoring for the transgene in the females of the brood colony can be made through real-time polymerase chain reaction, with results available within a day. Improvements to the genetic background of the mass reared strain can be made through occasional introduction of wild males into the system without compromising the essential qualities of the genetic sexing mechanism. Finally, once developed in a selected pest, this technology can be transferred to other lepidopteran species possessing the female-specific W chromosome by using similar transgene constructs.

The transgenic approach has two potential pitfalls: 1) there is no strong evidence that the transgene (N^{60g11}) will be expressed and exhibit the desired temperature lethality in female embryos, and 2) there are no data to predict the potential success of inserting the transgene into the W chromosome. The first concern could be overcome by identifying another DCLM. This will be facilitated by the recent report of a whole genome shotgun sequence for *B. mori* (Mita et al. 2004). Theoretically, the transgene insertion into the W chromosome is a matter of probability, which is dependent on the size of the W chromosome relative to the rest of the genome. Nevertheless, in most lepidopteran species examined, the W chromosome was found to consist largely of heterochromatin (Traut and Marec 1996, 1997), which may reduce the probability of an insertion event and/or silence the expression of the transgene. The latter phenomenon is well known from *Drosophila* as position-effect variegation (Schotta et al. 2003), although there are no reports of such effects in Lepidoptera. The screening process to establish the genetic sexing strain should reveal whether this is a serious problem.

Alternative Transgenic Approaches

If transgenesis is not accomplished by direct insertion on the W chromosome, but occurs on any other chromosome (preferably on the Z chromosome), then the segment containing the transgene can be translocated onto the W chromosome by standard genetic approaches (Marec and Mirchi 1990). A translocation of the transgene from the Z chromosome onto the W chromosome is preferred for two reasons. 1) A T(W;Z) translocation should occur at a higher frequency than a T(W;A) because in mature female pupae, which seem to be the best stage for irradiation, the W and Z pair in a sex chromosome bivalent (Marec and Mirchi 1990). 2) T(W;Z) translocations have been shown to improve pairing affinity of otherwise nonhomologous W and Z chromosomes (Marec and Traut 1994), whereas a T(W;A) translocation would probably result in the formation of a meiotic quadrivalent involving both sex chromosomes and two corresponding autosomes (Marec et al. 2001). Consequently, some sterility could be expected due to unbalanced segregation of the chromosomes involved in the quadrivalent. In the case of transgene insertion into the Z chromosome and its translocation onto the W chromosome, a similar scheme for composition of the genetic sexing strain can be used as presented in Fig. 2.

Transgenesis can be helpful even if an appropriate DCLM gene cannot be identified. For example, an automatic system for identifying and killing females can be developed based on the insertion of the fluorescent marker into the W chromosome. This approach could operate as an in-line optic system that screens egg sheets and physically removes or kills fluorescent eggs, although such systems based on individual observation are not optimal for mass rearing.

Potential Candidate for the Use of a Genetic Sexing Strain to Improve SIT

The codling moth is the key lepidopterous pest of most pome fruit (apple, pear, and quince) and some walnut orchards in the temperate regions of the world (Barnes 1991). The damage inflicted on fruit can be considerable with up to 80% of apples and 60% of pears infested on neglected apple and pear trees. The extensive use of organophosphate and other broad-spectrum insecticides to control the codling moth has resulted in the development of resistance and cross-resistance to these chemicals (Varela et al. 1993). Taking into account factors such as economic and global importance, mass rearing history, radiation biology, migration behavior, and potential for reinfestation, host range, national/international support, and the presence of monitoring tools for the most important lepidopterous pests, the codling moth is considered to be the best candidate for SIT application as part of an areawide integrated intervention approach.

In addition, the codling moth also seems to be a good candidate for a related suppression strategy, i.e., IS (LaChance 1985, Bloem et al. 1999).

After the successful implementation of a pilot SIT trial against the codling moth in 1976–1978 (Proverbs et al. 1982), an areawide operational intervention program was initiated in the Okanagan Valley, British Columbia, Canada, in 1992 (Bloem and Bloem 2000). A mass-rearing facility was constructed which had a production capacity of 15 million moths per week in 2002 (Lorne Tomlin, personal communication). Excellent results have been obtained in the 10 yr since operations were started in the first release zone. Insecticide use has been reduced by 82% from 18,903 kg in 1991 to 3,403 kg in 2001, and the percentage of orchards with no detectable level of codling moth damage at harvest has increased from 42% in 1995 to 91% in 1997 (Bloem et al. 2005).

Notwithstanding the success of the SIT program against codling moth in the Okanagan, there is a considerable potential to increase the efficiency of the technology by the development of genetic sexing strains. The benefits of the release of male-only strains compared with bisexual releases for the efficiency of SIT programs have been clearly documented with fruit flies (Rendon et al. 2004). In addition, releasing only male moths will eliminate the risk of releasing partially fertile females as part of an IS approach. It is therefore anticipated that the efficiency of both SIT and IS approaches for the control of codling moth populations could be significantly improved if genetic sexing is available.

As a result of the clear demonstration of the feasibility of using the SIT against the codling moth in the Okanagan program, the demand for the expansion of codling moth SIT to other countries (e.g., South Africa, Argentina, Brazil, and Chile) has increased dramatically in recent years.

Practical Requirements for the Development of Transgenic Sexing Strains in the Codling Moth

As mentioned above, a plasmid construct containing the *piggyBac* transposon with N^{60g11} in tandem with the *EGFP* marker gene under the *Actin A3* promoter is available (Fig. 3). Although preliminary experiments showed that it can be used for codling moth transgenesis (Ferguson et al. 2004), some improvements would be worthwhile. Although EGFP is useful for identifying transformants, it is a poor fluorescent marker in species such as the codling moth, which has excessive autofluorescence in the range used to visualize EGFP. In contrast, DsRed2 seems to be a better, more stable fluorescent marker (Horn et al. 2002), and the codling moth has little autofluorescence in this range.

Although it seems that the *B. mori Actin A3* (*BmA3*) promoter functions in the codling moth, it sometimes exhibits a chimeric fluorescence pattern in early larvae and adults (Ferguson et al. 2004). This chimeric expression also has been described in other species (Allen et al. 2004). However, the artificial eye- and neuron-specific promoter *3xP3* is a widely successful marker that promotes EGFP expression specifically in the insect eyes of all life stages (Horn and Wimmer 2000, Kokoza et al. 2001, Lorenzen et al. 2002, Thomas et al. 2002). We expect that the promoter would work in the codling moth, because the *BmA3* promoter already functions in this insect.

Because the W chromosome is heterochromatic, it may be necessary to use insulator elements in the transposon cassette. The insulators could prevent silencing effects of the W heterochromatin by establishing a higher order domain of chromatin structure in the insertion site. Numerous insulator sequences have been identified from *Drosophila* (Arnosti 2002), including one known from the *gypsy* retrotransposon with the requisite properties (Gdula et al. 1996). Other good candidates are the scs and scs' insulators that flank the *hsp70* gene in *Drosophila* (Kellum and Schedl 1991, 1992; Blanton et al. 2003). Last, if the N^{60g11} transgene will not work optimally in the codling moth, it may be necessary to identify other DCLMs.

Finally, in developing transgenic technology in the codling moth (or other pest insects), the absence of *Drosophila*-like genetic tools can be a disadvantage. For example, only one visible marker is currently available in this species (an autosomal mutation, golden moth; Hutt and White 1975). Identification of additional visible markers and/or molecular markers, especially on the sex chromosomes, would be useful.

In conclusion, the development of a practical, efficient, and reliable genetic sexing strain in the codling moth will greatly facilitate implementation of SIT/IS for this species. The functional strengths of the proposed scheme are that fitness of irradiated males is unimpaired by the genetic sexing mechanism because it is restricted to the female-specific W chromosome, and transgenic females are not released into the environment. Additional lines of defense against accidental transgenic release include the irradiation step, which will reduce fertility, and use of a cold sensitive lethal, which can eliminate transgenic females in the climate where this strategy will be used. The scheme for propagating the strain is genetically simple, in that under permissive conditions only one stock need be maintained, and visible markers (e.g., EGFP or DsRed2) are incorporated to monitor its genetic integrity. Furthermore, overall strain fitness can be enhanced by occasional outcrossing to wild males without compromising the genetic integrity of the stock. In contrast to the current genetic sexing Mediterranean fruit fly strains used for SIT, the proposed lepidopteran strain should be fully fertile and stable due to the absence of recombination between the W and Z chromosomes. However, as shown in Mediterranean fruit fly, strain stability under experimental laboratory conditions may not translate into similar stability under mass rearing conditions (Franz 2002).

Many of the assumptions inherent in this approach, especially regarding the overall behavior of transgenes, must still be experimentally verified. Concerns related to transgenic effects on organismal fitness have already been raised (Catteruccia et al. 2003, Irvin et al.

2004), and it may be necessary to produce several transgenic female lines from which a suitable one is selected for mass rearing facilities on the basis of appropriate fitness evaluations. Moreover, stability and expression of a transgene under conditions for mass rearing of these kinds of insects as well as their field behavior must be rigorously evaluated.

Despite these caveats, it should be emphasized that development of a genetic sexing strain according to the proposed scheme is technically feasible. With the exception of the function of a dominant cold sensitive lethal allele, the proof of principle for the main components of this scheme has already been established in Lepidoptera. These include 1) production of W-specific translocations and visible markers encompassing a balanced lethal system, both of which have been used for efficient and effective genetic sexing; 2) a system for transgenesis; and 3) release of irradiation-sterilized insects for population suppression. The novel aspects include inserting a transgene with a dominant cold-sensitive lethal allele on the W chromosome and its effectiveness in killing females. These are testable conditions that are presently under investigation.

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