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REVIEW ARTICLE

Use of a regulatory mechanism of sex determination in pest insect control

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Abstract

The sexual development of an insect is defined through a hierarchical control of several sex determining genes. Of these genes, *transformer* (*tra*) and *doublesex* (*dsx*) are well characterized and functionally conserved, especially *dsx*. Both genes are regulated at the transcriptional level through sex-specific alternative splicing. Incorporation of a genetically engineered sex-specific splicing module derived from these genes in transgenic systems, such as RIDL (release of insects carrying a dominant lethal), would allow the production of male-only insects for control programmes without any physical intervention.

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Introduction

In insects, sex determination is regulated through a cascade of gene regulation. A master switch at the top of the cascade decides the sex of each individual. This key switch varies among the different groups of insects. For example, in Drosophilids it is the number of X chromosomes (Erickson and Quintero 2007); in tephritids, a male factor linked to the Y chromosome (Willhoeft and Franz 1996; Dübendorfer *et al*. 2002); in moths, a female factor linked to the W chromosome (Masataka *et al*. 2002; Abe *et al*. 2008); in honeybees a complementary sex determiner (*csd*) gene; and in wasps, the haplo–diploid status of the egg in addition to maternally contributed factors (Cook 1993; Beye *et al*. 2003; Verhulst *et al*. 2010).

Though the primary signal for sex determination seems quite variable among different insects, lower down the cascade there are some conserved genes or functions. These include the *transformer* (*tra*) and *doublesex* (*dsx*) genes. Both of these genes were first identified in *Drosophila melanogaster* in classical genetic screens, loss-of-function mutants showing abnormalities in sexual differentiation (Baker and Ridge 1980). Homologues of *tra* have been found in other *Drosophila* species (O'Neil and Belote 1992), several tephritids including *Ceratitis capitata* (Pane *et al*. 2002) and *Bactrocera oleae* (Lagos *et al*. 2007), *Anastrepha* species (Ruiz *et al*. 2007), the blowfly *Lucilia cuprina* (Concha and Scott 2009), the housefly *Musca domestica* (Hediger *et al*. 2010) and the wasp *Nasonia vitripennis* (Verhulst *et al*. 2010). However, the primary sequence is not well conserved and so it remains difficult to identify *tra* homologues in divergent species, even when the genome sequence is available. In fact, the first non-*Drosophila* homologue was identified by microsynteny—proximity to *l(3)Ah*—rather than by homology (Pane *et al*. 2002), and others have also resorted to imaginative cloning strategies, such as identification based on clustered putative Tra/Tra-2 binding sites (Hediger *et al*. 2010). In contrast, *dsx* is relatively well conserved and homologues have been identified in a wide range of insects including *Bactrocera tryoni* (Shearman and Frommer 1998), *Megaselia scalaris* (Kuhn *et al*. 2000), *Bombyx mori* (Ohbayashi *et al*. 2001), *Antheraea assama* and *A. mylitta* (Shukla and Nagaraju 2010), *Musca domestica* (Hediger *et al*. 2004), *Anopheles gambiae* (Scali *et al*. 2005), *Ceratitis capitata* (Saccone *et al*. 2008) and *N. vitripennis* (Oliveira *et al*. 2009).

In *Drosophila*, the sex-specific alternative splicing of *tra* is regulated by *Sex-lethal* (*Sxl*). *tra* in turn regulates the sex-specific alternative splicing of *dsx*. The female-specific splicing form of *dsx* and, except in drosophilids, *tra* is achieved through the assembly of Tra/Tra2 complex on conserved splicing regulatory elements (Baker and Wolfner 1988; Bur-

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tis and Baker 1989; Sosnowski *et al*. 1989; Ohbayashi *et al*. 2001; Pane *et al*. 2002, 2005; Lagos *et al*. 2005; Scali *et al*. 2005; Oliveira *et al*. 2009; Concha and Scott 2009). *Sxl* has therefore been described as the 'master switch' for sex determination in *Drosophila*. Homologues of *Sxl* have been identified in different groups of insects including other dipterans (Saccone *et al*. 1998; Meise *et al*. 1998; McAllister and McVean 2000; Lagos *et al*. 2005), lepidopterans (Niimi *et al*. 2006), hymenopterans and coleopterans (Traut *et al*. 2006). However, the sex determination role of *Sxl* does not seem to be conserved or ancestral, rather it appears to be a recently acquired novel function restricted to a rather small group of insects including *Drosophila*. In nondrosophilids, *tra* autoregulates its own splicing in cooperation with the splicing factor transformer 2 (*tra-2*) (Salvemini *et al*. 2009; Hediger *et al*. 2010). This autoregulatory loop then epigenetically maintains the male or female state. This key epigenetic property, which is not shared by *Drosophila tra*, led Salvemini *et al*. (2009) to propose renaming *Cctra* as $Cctra^{ep}$ and that autoregulatory versions of tra should be considered orthologues of $Cctra^{ep}$ rather than of *tra*. Of course discussing the autoregulation begs the question of what establishes the male or female splicing pattern of *tra*, which is then maintained by this autoregulatory loop. This must be controlled, directly or indirectly, by the primary sex determination signal. However the mechanism(s) whereby this occurs are unknown. There are indications in medfly (*C. capitata*) that this might involve maternal contribution of female-type *tra* (mRNA or protein) driving female-type splicing of zygotic *tra*, inhibited in male embryos by the M-determining locus (Pane *et al*. 2002). As well as addressing fundamental questions of basic science, such as the genetic basis of sexual identity and differentiation, these studies provide molecular and genetic tools of potential value to applied scientists looking for new methods to control pest populations.

The sterile insect technique (SIT) is a species-specific and environmentally friendly method of insect biological control (Dyck *et al*. 2005; Alphey *et al*. 2010). This technique has been used to eliminate the New World screwworm, *Cochliomyia hominivorax*, from USA, Mexico and Libya (Lindquist *et al*. 1992), and also to control or eliminate medfly, *C. capitata*, in parts of Latin America (Hendrichs *et al*. 1995), and melon fly, *B. cucurbitae*, in Japan (Koyama *et al*. 2004). A newly developed variant of SIT known as RIDL (release of insects carrying a dominant lethal gene or genetic system) depends on the use of 'genetically sterile' insects engineered to carry a conditional dominant lethal (Thomas *et al*. 2000; Alphey and Andreasen 2002; Alphey *et al*. 2002, 2008). The inclusion of a sex-specific regulator to restrict the expression of the dominant lethal to females only would allow the elimination of females and release of only male insects without the need for any physical sex separation. Male-only releases can dramatically increase the efficiency of SIT-type control (Rendón *et al*. 2004).

Use of alternative splicing of sex determining genes in pest insect control

A bisex RIDL system containing a tetracycline-repressible positive feedback transactivator (tTA) was successfully constructed in *C. capitata* (Gong *et al*. 2005) and in *Aedes aegypti* (Phuc *et al*. 2007). In this system tTA acts not only as a transactivator but also as a lethal effector. Under restrictive conditions; namely in the absence of tetracycline, tTA accumulates in both sexes of the transgenic insect to levels that are lethal to immature stages. One feature of this molecular design is that it does not need a specific promoter derived from the target species, merely a minimal promoter used in conjunction with oligomerised *tet*O, the binding sequence of tTA. However, to use these insects in a 'male-only' release programme the females need to be removed by an independent method, which may be difficult for some insect species. It was therefore considered desirable to make the underlying molecular system female-specific. Though repressible female-specific lethality was previously achieved in *Drosophila*, and also later in *A. aegypti*, using a female-specific promoter as the source of sex specificity (Heinrich and Scott 2000; Thomas *et al*. 2000; Fu *et al*. 2010), it would not be straightforward to incorporate such a promoter into the 'positive feedback' system of Gong *et al*. (2005); one of the features of this system is that it does not depend on the use of a specific endogenous promoter. In principle, a sex-specific alternative splicing system offers an alternative route to achieve female-specific expression, without needing a female-specific promoter. Functional Tra protein is only produced by females as it is encoded by a splice variant exclusively produced in females. Indeed, the Tra promoter itself is not sex-specific, even though expression of functional Tra protein is mediated by this sex-specific alternative splicing mechanism. If this mechanism could be incorporated into the tTA transcript then it should possible to arrange that functional tTA protein is only produced in females; since the lethal effect depends on the production of large amounts of tTA protein this would render the system female-specific.

To add a sex selective component to the postitive feedback system of Gong *et al*. (2005), the first intron of the sex determining gene *Cctra* was inserted in the DNA sequence coding for tTA (see figure 1a). If this *Cctra* fragment is spliced in this context in the same way as in its native context, tTA production is only allowed when the intron is spliced in the female-specific form, as only in this variant is the continuous coding frame of tTA restored. Since the full splicing of this intron in its native gene is strictly confined to females (Pane *et al*. 2005), tTA expression was only expected in transgenic females. Analysis of tTA transcription in transgenic *C. capitata* showed a sex-specific pattern identical to that of the endogenous *Cctra* gene (Fu *et al*. 2007). The inserted intron spliced to generate three different transcripts of tTA: one female-specific (F1) and two nonsex-specific (M1 and M2) (figure 1). The female-specific

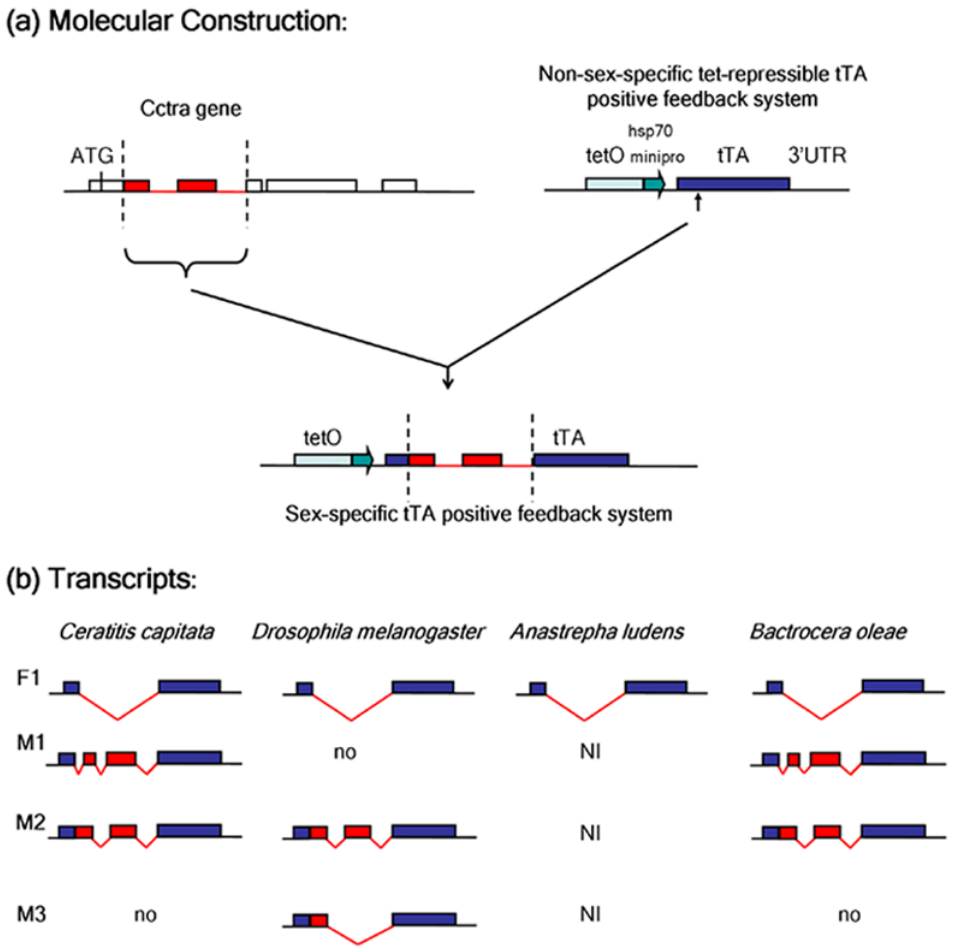

Figure 1. A sex-specific alternative splicing module for insect synthetic biology. (a) Insertion of appropriate sequence from *Cctra* in the tTA component of a non-sex-specific tetracycline-repressible positive feedback system. The arrow on tTA (blue box) indicates the point of *Cctra* intron insertion. (b) Alternative splicing of such a *Cctra*-tTA construct in transgenic insects of various species. F_1 is a female-specific transcript with the whole *Cctra* intron spliced out and, therefore, does produce a functional tTA. M1, M2 and M3 are non-sex-specific transcripts with some parts of the *Cctra* intron spliced out leaving non-coding exons (red boxes) which interrupt the reading frame for tTA and, therefore, do not produce functional tTA. There is no M1 transcript in transgenic *D. melanogaster* and no M3 in *C. capitata*. Non-sex-specific transcripts were not investigated (NI) in *A. ludens*.

transcript was the only one encoding a full tTA. As a consequence, all of the transgenic female progeny died as larvae or pupae when reared under restrictive conditions.

Transgenic lines with the same genetic sexing RIDL components were also generated in *Bactrocera oleae* (T. Ant, G. Fu, M. Koukidori and L. Alphey, unpublished data) and *Anastrepha ludens* (Koukidou *et al*. 2008), representing two other genera of tephritid fruit flies. When these transgenic insects had been reared under restrictive conditions, they all produced, as with *C. capitata*, male-only transgenic progeny. This means that the *Cctra* intron splices correctly to produce the F_1 transcript of tTA only in females (figure 1b). This suggests that this *tra* intron is functionally well conserved; this notion of functional conservation is supported by the similarly successful use of the equivalent *tra* fragment from *B. zonata* in a similar construct in *C. capitata* (K. Stainton, G. C. Condon, G. Fu and L. Alphey, unpublished data). Furthermore, constructs using the *Cctra* intron were tested in transgenic *D. melanogaster* (Fu *et al*. 2007). Despite the greater phylogenetic distance between *Drosophila* and the tephritids, female-specific lethality was still achieved. Molecular analysis showed that the *Cctra* sequence was correctly spliced in that the key 'female-specific' transcript was indeed only present in females, even though some differences were detected in the splicing of the non-sex-specific transcripts (figure 1b). This functional conservation contrasts strikingly with the relatively poor conservation of the primary sequence of Tra between these species. Further, there are key differences between the regulations of *tra* splicing in these two species; in *Drosophila* it is regulated by *Sxl* whereas in med-

fly it is regulated by *Cctra* itself. Part of the reason for this functional conservation may lie in presence of multiple sequences in the *Cctra* intron which correspond to the consensus binding sequence defined for *Drosophila* Tra/Tra-2 complex (Pane *et al*. 2002).

This use of a *Cctra*-derived alternative splicing module represents a step towards one of the key goals of synthetic biology—the construction and characterisation of discrete functional modules that can be combined to give predictable functions (Weber and Fussenegger 2009). Here, the positive feedback loop, the tetracycline-dependent switch and the sex-specific alternative splicing system each represent independent modules combined to give an outcome which, by design, represents the sum of the parts.

The sex-specific alternative splicing of the sex determining gene *dsx* provides another potential source of alternative splicing modules. *dsx* is highly conserved (Wilkins 1995) and so is relatively easy to isolate from any insect species. However, there are some potential complications. In *Drosophila*, the splicing regulatory elements appear to be dispersed in the exons flanking the sex-specifically splicing intron(s) (Sciabica and Hertel 2006), which would complicate its practical usage because the key regulatory sequences obviously have to be included in any sex-specific 'splicing-module' to maintain their sex-specific regulation. Few *dsx* homologues have been characterised in detail, and this issue may not apply to all. However, it is clear that the primary transcripts of many *dsx* genes are rather large, due to the presence of long introns. This would make it difficult to develop a compact alternative-splicing module based on unmodified *dsx*. One approach to overcome this problem is to develop 'minigene' versions, in which much of the intronic sequence is deleted. Naturally, functional elements essential for the alternative splicing would need to be retained and these may not readily be identified by bioinformatic approaches exclusively, though experimental approaches to this problem are relatively trivial. Functional minigenes have been successfully derived from the *dsx* gene of *Bombyx mori* and of other insect species. (Funaguma *et al*. 2005; G. Labbe, T. Dafa'alla and L. Alphey, unpublished data).

One potential issue that might arise through the use of such alternative splicing modules in insect synthetic biology is interference with endogenous processes through the use or sequestration of limiting regulatory factors. One might then see an effect on sexual differentiation and/or saturation of the ability to produce female-specific transcripts in the engineered system. However, no such effect was observed in the system of Fu *et al*. (2007), suggesting that the regulatory factors involved in the alternative splicing of sex determination genes, especially in *tra* and *dsx* splicing, are available in abundance and the sex-specific splicing system of *tra* is not easily saturated. This feature makes the application of these factors to regulate the expression of a transgene(s) very practical.

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