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Effect of the gene *transformer of Anastrepha* on the somatic sexual development of *Drosophila*

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ABSTRACT The gene *transformer (tra)* is the key regulatory memory device for sex determination in tephritid insects. The present manuscript addressed the question about the functional conservation of the tephritid *Anastrepha* Transformer protein to direct somatic sexual development in *Drosophila* (Drosophilidae). The *transformer* cDNA of *Anastrepha* encoding the putative full-length Tra protein was cloned in *pUAST* and introduced into *Drosophila melanogaster*. To express this protein, the GAL4-UAS system was used. The *Anastrepha* Tra protein induced the female-specific splicing of both *dsx* and *fru* pre-mRNAs in *Drosophila* XY male flies, so that these became transformed into females, though this transformation was incomplete (the sexually dimorphic foreleg basitarsus and the external terminalia were monitored). It was found that the degree of female transformation directly depended on the dose of *Anastrepha tra* and *Drosophila transformer-2 (tra-2)* genes, and that the *Anastrepha* Tra-*Drosophila* Tra2 complex is not as efficient as the *Drosophila* Tra-Tra2 complex at inducing the female-specific splicing of *Drosophila dsx* pre-mRNA. This can explain why the *Anastrepha* Tra protein cannot fully substitute for the endogenous *Drosophila* Tra protein.

KEY WORDS: *Anastrepha, Drosophila, transformer, sexual development*

Introduction

The genetic basis of sex determination in *Drosophila melanogaster* is understood in fine detail. The epistatic relationships between the sex determination genes have revealed them to be subject to hierarchical interaction (reviewed in Sánchez *et al.,* 2005; Sánchez 2008). Briefly, the gene *Sxl*, which is at the top of this cascade, acts as the memory device for female sexual development via its auto-regulatory function: the Sxl protein participates in the female-specific splicing of its own pre-mRNA (Cline 1984; Bell *et al.,* 1991). The downstream target of *Sxl* is the gene *transformer (tra)*. A transcript found in both males and females encodes a non-functional truncated Tra protein, and a female-specific transcript encodes the functional Tra protein (Boggs *et al.,* 1987; McKeown *et al.,* 1987; Nagoshi *et al.,* 1988; Belote *et al.,* 1989; Inoue *et al.,* 1990). The Tra product and the product of the constitutive gene *transformer-2 (tra-2)* (Goralski *et al.,* 1989; Amrein *et al.,* 1990) control the sex-specific splicing of the pre-mRNA of the gene *doublesex (dsx)*, which is transcribed in both sexes (Burtis and Baker 1989; Hoshijima *et al.,* 1991; Hedley and Maniatis 1991; Ryner and Baker 1991; Tian and Maniatis 1993; Hertel *et al.,* 1996). In females, the Tra-Tra2 complex directs the splicing of the *dsx* pre-mRNA according to the female mode, giving rise to the female DsxF protein that promotes female sexual development. In males, in which no functional Tra protein is available, the *dsx* pre-mRNA follows the default male mode of splicing, giving rise to the mature *dsxM* mRNA, which produces male DsxM protein. This promotes male sexual development.

The Tra-Tra2 complex also controls the sex-specific splicing of pre-mRNA from the P1-promoter of *fruitless (fru)*, a gene involved in the male sexual development of the central nervous system (CNS) (Rideout *et al.,* 2007). In females, the binding of this complex to their target sites in the female-specific exon promotes its inclusion into the mature mRNA. This exon contains translation-stop codons so that in females no FruM protein is produced. In males, in which no Tra protein is available, the female-specific

Abbreviations used in this paper: dsx, doublesex; fru, fruitless; tra, transformer.

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exon is spliced out and the FruM protein is produced (Ryner *et al.,* 1996; Heinrichs *et al.,* 1998; Goodwin *et al.,* 2000; Billeter *et al.,* 2006).

The gene *Sxl* has been characterised in the tephritids *Ceratitis capitata* (Medfly) (Saccone *et al*., 1998) and *Bactrocera oleae* (olive fly) (Lagos *et al.,* 2005). In these species, *Sxl* is not regulated in a sex-specific fashion, and therefore the same *Sxl* transcript encoding the functional Sxl protein is found in both males and females. Thus, *Sxl* does not appear to play in the tephritids the key discriminating role in sex determination that it plays in *Drosophila*.

Though it has recently been reported that, in *Drosophila*, the gene *tra* has a minor role in *Sxl* auto-regulation - probably a relic of the evolutionary transition from *tra* to *Sxl* as the master regulatory gene for sex determination (Siera and Cline 2008) – it is normally considered as simply another gene in the sex determination genetic cascade. In the tephritids, however, *tra* plays a key regulatory role, acting as the memory device for sex determination via its auto-regulatory function (Pane *et al.,* 2002). The gene *tra* has been characterised in the tephritids *Ceratitis capitata* (Pane *et al.,* 2002), *Bactrocera oleae* (Lagos *et al.,* 2007) and in twelve *Anastrepha* species (Ruiz *et al.,* 2007a). It is constitutively expressed in both sexes and its primary transcript shows sex-specific alternative splicing. It has male-specific exons that contain translation stop codons. The incorporation of these exons into the mature *tra* mRNA in males determines that a truncated, non-functional Tra protein be produced. In females, the male-specific exons are spliced out owing to the presence of Tra protein. The presence of putative Tra-Tra2 binding sites in the male-specific exons and in the surrounding introns suggests that the tephritid Tra and Tra2 proteins form a complex, the binding of which to their target sequences prevents the male-specific exons from becoming incorporated into mature *tra* mRNA.

In the tephritids, the gene *tra* controls also the sex-specific splicing of the *dsx* primary transcript (Pane *et al.,* 2002; Lagos *et al.,* 2007; Salvemini *et al.,* 2009). The gene *dsx* has been characterised in the tephritids *Bactrocera tryoni* (Shearman and Frommer 1998), *Bactrocera oleae* (Lagos *et al.,* 2005), *Bactrocera dorsalis* (Chen *et al.,* 2008), *Ceratitis capitata* (Saccone *et al.,* 2008), and in eleven *Anastrepha* species (Ruiz *et al.,* 2005; 2007b). The molecular organisation of the *dsx* ORF varies among these insects, but in all cases *dsx* encodes male- and female-specific RNAs that encode putative male- and female-specific Dsx proteins that share the N-terminal region but which differ in their C-terminal regions (as in *Drosophila*). The protein finally produced depends on the result of the sex-specific splicing of the primary transcript. In all these tephritids, putative Tra-Tra2 binding sites are found in the female-specific exon, suggesting that, like in *Drosophila*, male-specific splicing represents the default mode and that female-specific splicing requires the Tra protein (which is only present in females). Finally, the *tra-cDNA* of *Ceratitis (Cctra)* encoding the putative full-length Tra protein transforms transgenic *D. melanogaster* males into females, though this transformation appears to be incomplete (Pane *et al.,* 2005).

The present manuscript addressed the question about the functional conservation of the tephritid *Anastrepha* Transformer protein to direct the sexual development in *Drosophila* (Drosophilidae). To this respect, the *tra-cDNA* of *Anastrepha obliqua* (*Aotra)* encoding the putative full-length Tra protein was introduced into *D. melanogaster*, and its effect on sexual development in these transgenic flies recorded.

Results and Discussion

The GAL4-UAS system was used to analyse the effect of *Aotra* in *Drosophila*, in which *Aotra* was linked to UAS sequences. The used GAL4 drivers direct expression of *Aotra* ubiquitously.

The sexual transformation was monitored in the sexually dimorphic male foreleg basitarsus and the external terminalia. The male foreleg basitarsus possesses the sex comb structure (SC in Fig. 1A), which is absent in females (Fig. 1B). The most conspicuous sexual dimorphic regions of the fly are the external terminalia, which derive from the genital disc (reviewed in Sánchez and Guerrero 2001; Christiansen *et al.,* 2002; Sánchez *et al.,* 2005). This is composed of two genital primordia plus the anal primordium. In both sexes, only two of these three primordia develop to form the adult terminalia. The anal primordium develops in both males and females, but form either the male or female analia. However, only one of the two genital primordia develops,

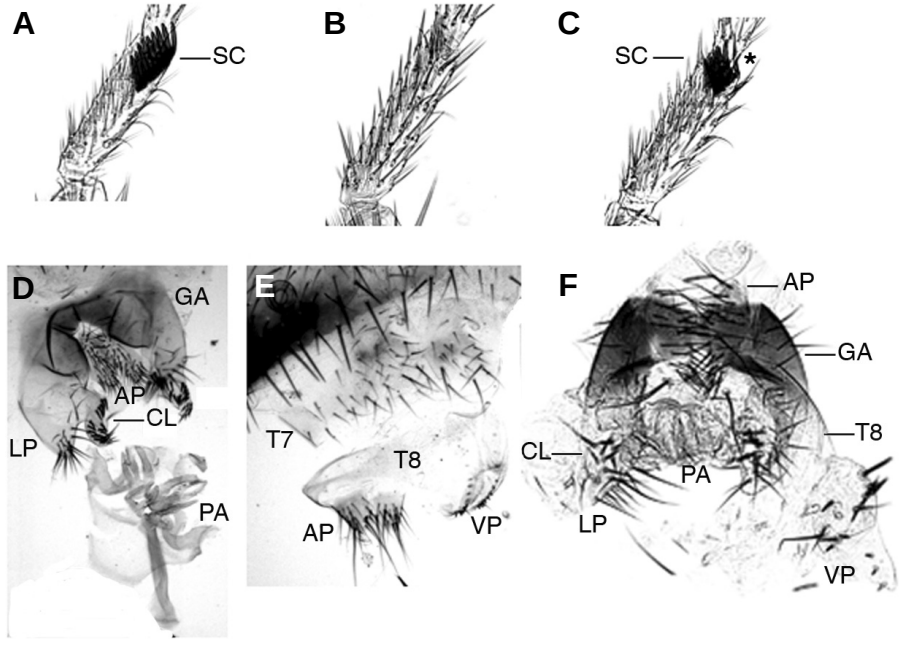

Fig. 1. Morphological analysis of *Aotra* transgenic *Drosophila* flies. (A,D) *Wild type male.* **(B,E)** *Wild type female.***(C)** *Transgenic XY male* ywTAo#1/Y; arm-GAL4/+. *Notice the reduction of the sex comb size and the intersex nature of the distal bristle (asterisk) in the sex comb.* **(F)** *Transgenic pseudomale* ywTAo#1/w; arm-GAL4/CyO*;* tra^{v1}/Df(3L)E52, tra[-]. *Notice the presence of female and male genital structures and the intersex nature of the anal plates. AP, anal plates; CL, claspers; GA, genital arch; LP, lateral plates, PA, penis apparatus comprising penis proper and hypandrium; SC, sex comb; T8, tergite 8; VP, vaginal plates.*

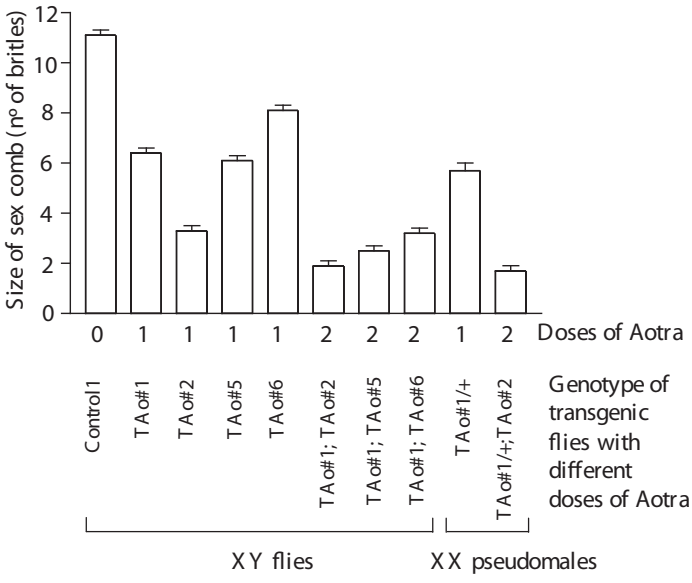

Fig. 2. Size (number of bristles) of the sex comb in *Aotra* transgenic *Drosophila* flies. *The bars in the histograms represent the 95% confidence limits. The control was* ywTAo#1/Y; CyO/+ *males (cross I).* TAo#1 *stands for* ywTAo#1/Y; arm-GAL4/+ *(cross I);* TAo#2 *stands for* yw/Y; arm-GAL4/TAo#2 *(cross II);* TAo#5 *stands for* yw/Y; arm-GAL4/TAo#5 *(cross III);* TAo#6 *stands for* yw/Y; arm-GAL4/TAo#6 *(cross IV);* TAo#1; TAo#2 *stands for* ywTAo#1/Y; arm-GAL4/TAo#2 *(cross V);* TAo#1; TAo#5 *stands for* ywTAo#1/Y; arm-GAL4/TAo#5 *(cross VI);* TAo#1; TAo#6 *stands for* ywTAo#1/Y; arm-GAL4/TAo#6 *(cross VII); XX pseudomale* TAo#1/+ *stands for* ywTAo#1/w; arm-GAL4/+; tra^{v1}/tra[-] *(cross VIII); XX pseudomale* TAo#1/+; TAo#2 *stands for* ywTAo#1/w; arm-GAL4/TAo#2; tra^{v1}/tra[-] *(cross IX).*

forming either the male (Fig. 1D) or female (Fig. 1E) adult genitalia. The taken route depends on the genetic sex of the fly, i.e., the production of either the female DsxF or male DsxM proteins. When both proteins are present, the sexually dimorphic structures follow an intersex course of development, which in the case of the genital disc is characterised by the presence of incomplete male and female genital structures and the intersex phenotype of the analia.

A reduction was seen in the number of bristles forming the male sex comb structure in the foreleg basitarsus of *Aotra* transgenic XY males, this reduction being higher when increasing the doses of the *Aotra* transgene (Fig. 2). In some cases, intersexual bristles were found between the sex comb-like bristles (asterisk in Fig. 1C). In a very few cases, in which no sex comb-like bristles were found, the last transverse row was usually only partially rotated.

With respect to the external terminalia, both male and female genital derivatives were found. These terminalia comprise the female 8th tergite and vaginal plate structures, plus the male genital arch, lateral plates, claspers and penis apparatus (Table 1 and Fig. 1F). The anal plates were always intersexual. Increasing the doses of the *Aotra* transgene led to a reduction in the frequency of male structures, and a corresponding increase in the frequency of female structures – although complete female transformation was never observed. Similar findings were made when the effect of the *Aotra* transgene was analysed in XX pseudomales homozygous for the endogenous *tra* gene (Fig. 2 and Table 1).

In the XY flies expressing the *Aotra* transgene, female structures were expected due to the presence of DsxF. This expectation was confirmed by RT-PCR analysis, which revealed the existence of *dsxF* mRNA isoform in these transgenic flies. However, the *dsxM* mRNA isoform was still present, thus explaining their intersex phenotype (Fig. 3A). The sex-specific splicing of the *fru* pre-mRNA was also monitored in the transgenic XY flies. RT-PCR analysis showed that, in these males, the female-specific–*fru* mRNA was produced together with the male mRNA (Fig. 3B). Since the endogenous *Drosophila tra* mRNA found in the transgenic flies was only the male-specific isoform (Fig. 3C), which encodes a truncated, non-functional Tra protein, it can be concluded that the female-specific splicing of both *dsx* and *fru* pre-mRNAs observed in the transgenic flies is due to the direct function of the *Anastrepha* Tra protein on these primary transcripts, and not to an indirect effect of the endogenous *Drosophila tra* gene. Negative controls in all these PCR reactions did not produce amplicons (see Materials and Methods).

The *Drosophila* Tra protein seems to lack an RNA binding domain, thus its influence in female development is exerted at the level of its interaction (through their SR domains) with other proteins carrying RNA-binding domains, such as Transformer-2 (reviewed in Black 2003). Since the transgenic *Anastrepha* Tra protein does not have a recognisable RNA-binding domain either, the question arises as to whether this protein also needs to interact with the endogenous *Drosophila* Tra2 protein to direct the female development of *Drosophila* transgenic flies. To address this question, both *Aotra* transgenic XY and XX *Drosophila* flies mutant for *tra-2* were produced (see cross X in Materials and Methods). Both *ywTAo#1/Y; tra-2^{B}/ Df(2L)trix, tra-2[-]; da-GAL4/+* males and *ywTAo#1/w; tra-2^{B}/ Df(2L)trix, tra-2[-]; da-GAL4/+* pseudomales showed the normal male morphology (data not

TABLE 1

FREQUENCY OF EXTERNAL STRUCTURES IN THE TERMINALIA OF *D. MELANOGASTER* FLIES EXPRESSING THE *ANASTREPHA* TRA FEMALE PROTEIN

Cross	Genotype	Frequency of female genital structures		Frequency of male genital structures				Anal Plates
		T8	VP	GA	LP	CL	PA	
I	*ywTAo#1/Y; arm-GAL4/+* (27)	1.0	1.0	1.0	0.96	0.7	1.0 (red)	Intersexual 1,0
II	*yw / Y; TAo#2 / arm-GAL4/+* (24)	1.0	1.0	1.0	0.96	0.83	1.0 (red)	Intersexual 1,0
III	*yw / Y; TAo#5 / arm-GAL4/+* (23)	1.0	1.0	1.0	1.0	0.96	1.0 (red)	Intersexual 1,0
IV	*yw/Y; TAo#6 / arm-GAL4/+* (25)	1.0	1.0	1.0	1.0	1.0	1.0 (red)	Intersexual 1,0
V	*ywTAo#1/Y; TAo#2 / arm-GAL4* (26)	1.0	1.0	0.46	0.42	0.35	1.0 (red)	Intersexual 1,0
VI	*ywTAo#1/Y; TAo#5 / arm-GAL4* (28)	1.0	1.0	0.43	0.39	0.07	1.0 (red)	Intersexual 1,0
VII	*ywTAo#1/Y; TAo#6 / arm-GAL4* (33)	1.0	1.0	0.76	0.64	0.54	1.0 (red)	Intersexual 1,0
VIII	*ywTAo#1/+; arm-GAL4/+; tra[-]/tra[-]* (32)	1.0	1.0	1.0	0.95	0.86	1.0 (red)	Intersexual 1,0
IX	*ywTAo#1/+; TAo#2/arm-GAL4; tra[-]/tra[-]* (37)	1.0	1.0	0.49	0.41	0.24	1.0 (red)	Intersexual 1,0

The number in parenthesis following the genotype indicates the number of analysed flies. Frequency refers to the presence of the corresponding structure. Symbols: CL, clasper; GA, genital arch; LP, lateral plates; PA, penis apparatus comprising the penis proper and hypandrium (red indicates reduced PA); T8, 8th tergite; VP, vaginal plates.

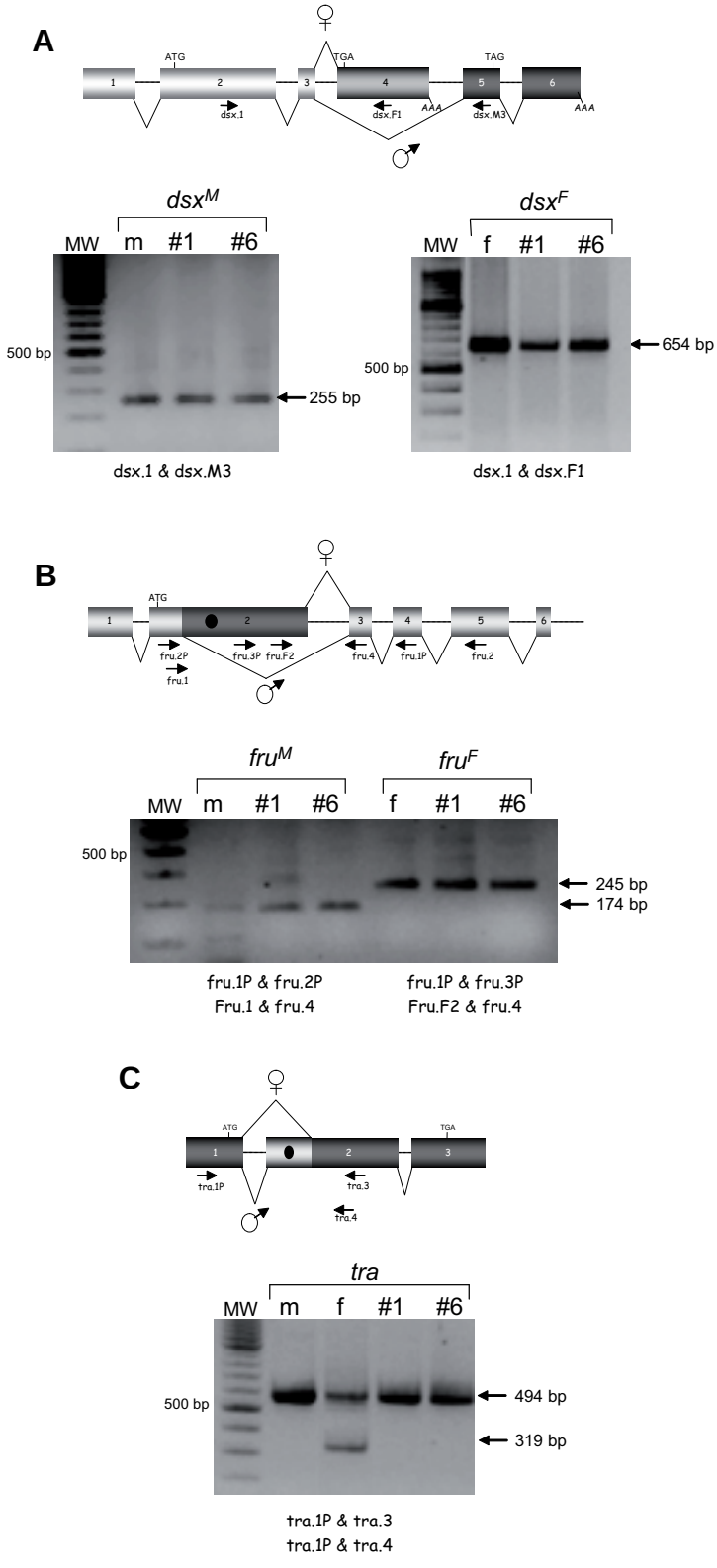

Fig. 3. Expression of *dsxF* (A), *fruF* (B) and *tra* (C) mRNAs in *Aotra* transgenic *Drosophila* XY flies. *The "m" and "f " letters stand for wild type male and female, respectively, whereas #1 and #6 stand for the XY flies of genotype* ywTAo#1/Y; arm-GAL4/+ *(cross I) and* yw/Y; arm-GAL4/ TAo#6 *(cross IV). The dot indicates the translation stop codon.*

shown), indicating that the *Anastrepha* Tra protein requires the endogenous *Drosophila* Tra2 protein to exerts its function in female development. This further suggests that both AoTra and DmTra2 proteins interact to form a complex.

The question naturally arises as to whether the incomplete female transformation observed in transgenic *Drosophila* flies is due to a low efficiency of the AoTra-DmTra2 complex to induce the female-specific splicing of the *dsx* pre-mRNA. This possibility was examined by analysing the structures of the external adult terminalia and the size of the sex comb in XY male flies expressing the transgenic AoTra protein and carrying either one or two doses of the endogenous *Drosophila tra-2* gene. XY male flies of genotypes *ywTAo#1/Y; arm-GAL4 tra-2[+]/tra-2[+]*(control males) and their brothers *ywTAo#1/Y; arm-GAL4 tra-2[+]/tra-2[-]*(experimental males) (cross XI in Materials and Methods) were raised at 25°C and the size of their external female and male genital structures as well as that of the sex comb monitored. Reducing the dose of *tra-2* led to a significant reduction in the size of the female genital structures (t test, $P<0.0001$) as well as a significant increase in the size of the male genital structures (t test, $P<0.0001$) in males carrying a single dose of endogenous *tra-2*: female vaginal plates 17.3 ± 1.2 vaginal teeth in the controls, compared to 4.0 ± 0.5 vaginal teeth in the experimental males; male genital arch 6.7 ± 0.5 bristles in the controls compared to 12.0 ± 0.4 bristles in the experimental males; male lateral plate 26.1 ± 1.3 bristles in the controls compared to 38.5 ± 0.7 bristles in the experimental males; and male clasper 19.2 ± 1.2 in the controls compared to 34.0 ± 0.9 in the experimental males (n° of control males = 42, n° of experimental males = 31). Similarly, flies with two doses of *tra-2* showed a significant reduction in the size of the sex comb (t test, $P<0.0001$) compared to flies carrying a single dose of *tra-2*: control males 6.0 ± 0.3 sex comb bristles, experimental males 7.7 ± 0.1 sex comb bristles. These results are explained by the smaller amount of *dsx* pre-mRNA present following female-specific splicing in the experimental males (with one dose of *tra-2* compared to two in the controls). This might be due to a reduced efficiency of the AoTra-DmTra2 complex to induce the female-specific splicing of the *dsx* pre-mRNA. This hypothesis was tested at the molecular level.

Transgenic XY brothers expressing the *Aotra* transgene and carrying either two doses (control males, M1) or one dose (experimental males, M2) of the endogenous *tra-2* gene were produced at 25°C (see cross XI in Materials and Methods). As a control, *D. melanogaster* XX sisters carrying different doses of the endogenous *tra-2* and *tra* genes were also produced at 25°C (see cross XII in Materials and Methods): F1 females carried two doses of *tra-2* and of *tra*, F2 females carried two doses of *tra-2* and one dose of *tra*, F3 females carried one dose of *tra-2* and two doses of *tra*, and F4 females carried one dose of *tra-2* and of *tra*. The presence of the *dsxF* mRNA isoform in these males and females was monitored by RT-PCR. The primers dsx1P (in the common exon 3) and dsxF1 (in the female-specific exon 4) were used for the PCR reaction (see Fig. 4A). The *Drosophila* sisters (F1-F4) and the transgenic *Drosophila* brothers (M1-M2) showed two amplicons, the lower band having the expected size for the mature *dsxF* mRNA isoform. Both amplicons were cloned and sequenced. It was thus confirmed that the smaller amplicon corresponded to standard, mature *dsxF mRNA*, whereas the second corresponded to a *dsxF* mRNA isoform that retains intron

3 between the common exon 3 and the female-specific exon 4. The signal for this latter amplicon was more intense in the transgenic M1 and M2 flies than in the F1, F2, F3 and F4 flies, suggesting differences in the splicing efficiency of the AoTra-DmTra2 and *Drosophila* DmTra-DmTra2 complexes. To better compare the efficiency of these two complexes in inducing the female-specific splicing of the endogenous *dsx* pre-mRNA, quantitative analysis of the female-specific splicing of this pre-mRNA in the *Aotra* transgenic *Drosophila* XY brothers M1 and M2, and in the *Drosophila* sisters F1, F2, F3 and F4, was performed. Five PCRs were performed for each of these brothers and sisters using the pair of primers dsx1P and dsxF1. The relative abundance of the amplicon corresponding to the *dsxF* mRNA isoform that retains intron 3 was then monitored (for details see legend to Fig. 4). Fig. 4B shows that the relative abundance of this non-standard *dsxF* mRNA isoform is very low and does not significantly vary among the four *Drosophila* sister types with different doses of *tra* and *tra-2* (P=0.1231), but a significant increase in this incompletely spliced *dsxF* mRNA isoform was seen, however, among the *Drosophila* and the *Aotra* transgenic M1 and M2 flies (P<0.0001) (one way ANOVA test). This increase was significantly higher in the transgenic M2 males (one dose of *tra-2*) than the M1 males (two doses of *tra-2*) (t test P=0.0012). Negative controls in all these PCR reactions did not produce amplicons (see Materials and Methods).

The considerable retention of intron 3 by the *Aotra* transgenic XY flies does not appear to be the consequence of any general trouble in the splicing of *dsx* pre-mRNA. PCR involving the pair of primers dsx1P (in common exon 2) and dsxF2 (in female-specific exon 4) produced the amplicon corresponding to the standard female-fusion of exons 2, 3 and 4, and a significant amount of the atypical amplicon carrying intron 3 as well. The *Drosophila* sisters F1 to F4 showed only traces of this aberrant amplicon (Fig. 4C). This indicates that intron 2 (and probably also intron 1) is normally spliced in the *Aotra* transgenic XY flies and that the anomalous splicing affects only intron 3. It should be remembered that the binding of the Tra-Tra2 complex to the female-specific exon 4 eliminates intron 3 from the *dsx* pre-mRNA in the female-specific splicing mode. Negative controls in all these PCR reactions did not produce amplicons (see Materials and Methods).

Collectively, these results indicate that the AoTra-DmTra2 complex appears to be not as efficient as the *Drosophila* Tra-Tra2 complex at inducing female-specific splicing of the *dsx* pre-mRNA. This can explain that the *Anastrepha* Tra protein cannot fully substitute for the endogenous *Drosophila* Tra protein, and then the incomplete transformation of transgenic *Aotra Drosophila* XY males into females. This further suggests that the interaction between the *Anastrepha* Tra protein and the *Drosophila* Tra2 protein might be affected as a consequence of changes accumulated in these proteins after the *Anastrepha* and *Drosophila* phylogenetic lineages separated. It cannot be ruled out, however, that the AoTra-DmTra2 complex shows less efficient binding to its target sequences in the *Drosophila*"*dsx* pre-mRNA than the *Drosophila* Tra-Tra2 complex. The expression of *Ceratitis* Tra protein in transgenic *Drosophila* XY male flies induced as well the female-specific splicing of both *dsx* and *fru* pre-mRNAS, but it fails to impose them a complete female transformation (Pane *et al.* 2005). This failure could also be due to a partially ineffective interaction between the *Ceratitis* Tra protein and the *Drosophila* Tra2 protein, since the *Anastrepha* Tra and *Ceratitis* Tra proteins show a high degree of similarity

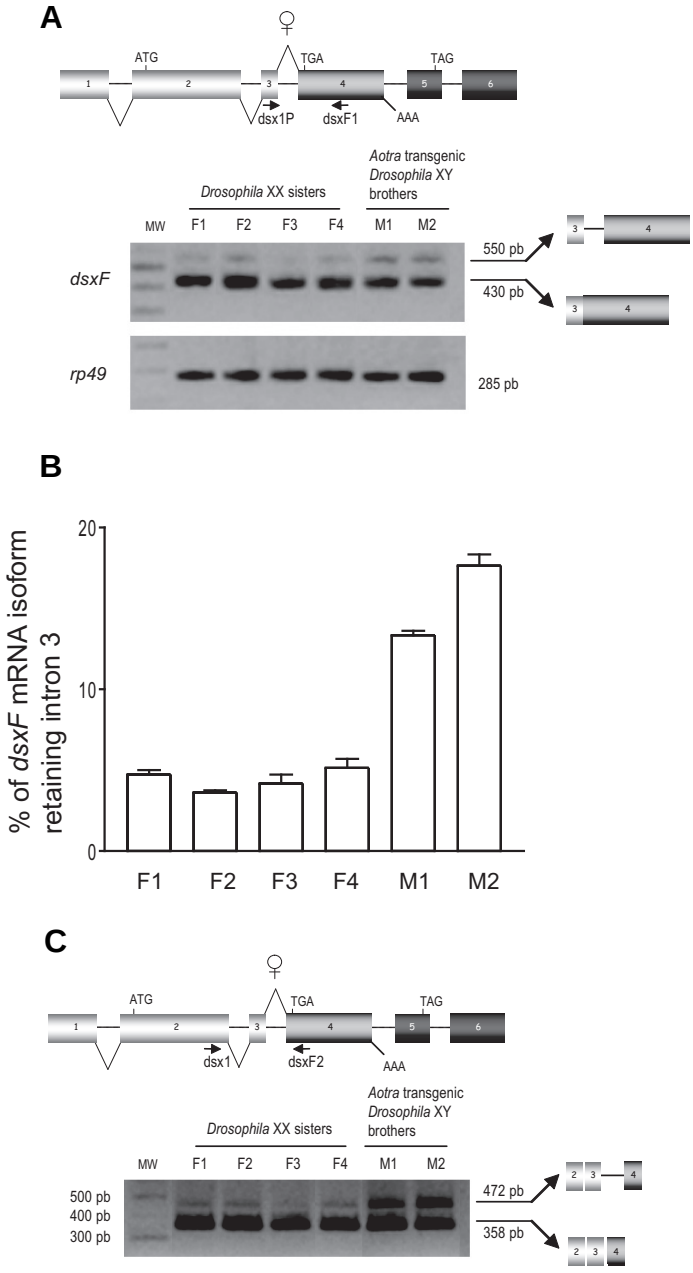

Fig. 4. Comparison of the splicing efficiency between the AoTra-DmTra2 and DmTra-DmTra2 complexes. *M1 and M2 stand for* Aotra *transgenic* Drosophila *XY flies with two or one doses of the endogenous* tra-2 *gene, and F1 to F4 for* Drosophila *females with different doses of* tra-2 *and* tra *genes. Full genotypes: M1,* ywTAo#1/Y; arm-GAL4[w^+]/CyO; *M2,* ywTAo#1/Y; arm-GAL4[w^+]/tra-2^B*; F1,* yw; CyO/+; TM3,Sb/+*; F2,* yw; CyO/+; tra^{v1}/+*; F3,* yw; tra-2^B/+; TM3,Sb/+*, and F4,* yw; tra-2^B/+; tra^{v1}/+*. The bands of the corresponding amplicons were measured with the ImageJ programme. As a control, the splicing of the* rp49 *gene that encodes for the constitutive ribosomal protein 49 (Ramos-Onsins* et al.*, 1998) was monitored.*

(Ruiz *et al.* 2007a). Further molecular analyses need to be performed to study the putative molecular co-evolution of these sex-determining Tra and Tra-2 proteins.

Materials and Methods

Flies and crosses

Flies were cultured on standard food. For the description of the mutant alleles and GAL4 constructs see Lindsley and Zimm (1992) and FlyBase. Flies used for the analysis of the adult forelegs and external terminalia were kept in a mixture of ethanol:glycerol (3:1) for several days. They were then macerated in 10% KOH at 60°C for 15 min, thoroughly washed with water, and mounted in Faure's solution for inspection under a compound microscope. *TAo* stands for *UAS::Aotra*-cDNA. The crosses were:

(I) Females *ywTAo#1* and males *w; arm-GAL4[w$^+$]/CyO*.

(II) Females *yw; TAo#2* and males *w; arm-GAL4[w$^+$]/CyO*.

(III) Females *yw; TAo#5* and males *w; arm-GAL4[w$^+$]/CyO*.

(IV) Females *yw; TAo#6* and males *w; arm-GAL4[w$^+$]/CyO*.

(V) Females *ywTAo#1; TAo#2* and males *w; arm-GAL4[w$^+$]/CyO*.

(VI) Females *ywTAo#1; TAo#5* and males *w; arm-GAL4[w$^+$]/CyO*.

(VII) Females *ywTAo#1; TAo#6* and males *w; arm-GAL4[w$^+$]/CyO*.

(VIII) Females *ywTAo#1; tra^{v1}/TM3,Sb* and males *w/B^{S}Y; arm-GAL4[w$^+$]/CyO; Df(3L)E52, tra[-]/MKRS,Sb*.

(IX) Females *ywTAo#1; TAo#2/CyO; tra^{v1}/MKRS,Sb* and males *w/B^{S}Y; arm-GAL4[w$^+$]; Df(3L)E52, tra[-]/MKRS,Sb*.

(X) Females *ywTAo#1; tra-2^B/CyO* and males *w/Y; Df(2L)trix, tra-2[-]/CyO; da-GAL4[w$^+$]/MKRS,Sb*.

(XI) Females *ywTAo#1; tra-2^B/CyO* and males *w/Y; arm-GAL4[w$^+$]*

(XII) Females *yw; tra^{v1}/TM3,Sb* and males *yw/B^{S}Y; tra-2^B/CyO*.

In the analysis of the requirement of *Drosophila tra-2* gene for the function of *Aotra* in *Drosophila*, the *da-GAL4* driver (located in the third chromosome) was used instead of *arm-GAL4* (located in the second chromosome). In this way, we could generate *Drosophila* transgenic flies mutant for *tra-2* (located in the second chromosome) without the synthesis of a recombinant chromosome carrying the *tra-2* mutation and the *arm-GAL4* driver.

Molecular analyses

Total RNA extracts from frozen adults were prepared using the Ultraspec-II RNA isolation kit (Biotecx) following the manufacturer's instructions. For analysis of the *fru* gene, the total RNA used was taken from frozen heads. Five micrograms of total RNA from each sample were reversed transcribed with Superscript (Invitrogen) following the manufacturer's instructions. Reverse transcription reactions were performed with an oligo-dT, except for *fru*, for which primer Fru2 was used. Two percent of the synthesised cDNA was amplified by PCR; 10% of the amplified product was kept for further re-amplification as needed. All amplicons were analysed by electrophoresis in agarose gels. In all cases, PCRs on RNA samples were performed to guarantee that they were not contaminated with genomic DNA (negative controls of PCR reactions).

For the construction of the *Aotra* transgene, the *tra* ORF of *Anastrepha obliqua* was amplified by RT-PCR. The PCR reaction was performed using primers Tra-Ao23 and Tra-Ao25. The resulting amplicon was cloned in *pUAST*. The microinjections for generating the *TAo* transgenic *D. melanogaster* lines were performed by Genetic Services (Sudbury, MA, USA). To ascertain that each transgenic line was carrying the correct transgene, PCR on genomic DNA was used to amplify the whole transgene and the amplicons were cloned and sequenced. The sequences of the primers are shown in the Supplementary Table S1. As expected, none of the *Aotra* transgenic *Drosophila* lines expressed the transgene in the absence of GAL4. If a basal expression of *Aotra* existed this would be unimportant since XY flies with one, two or three doses of this transgene are normal, fertile males.

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