Masculinization of XX Drosophila transgenic flies expressing the Ceratitis capitata Doublesex^M isoform

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ABSTRACT The Doublesex (DSX) transcription factor regulates somatic sexual differentiation in Drosophila melanogaster. Female and male isoforms (DSX^F and DSX^M) are produced due to sex-specific RNA splicing. Here we show that in the distantly related dipteran Ceratitis capitata, the DSX^M male-specific isoform is conserved and able to induce masculinization of both somatic and germline tissues when ectopically expressed in XX Drosophila transgenic individuals.

KEY WORDS: Ceratitis, sex determination, DSX^M isoform

Introduction

In the genetic model system Drosophila melanogaster, the doublesex gene (dsx) constitutes the terminal effector of the sex determination cascade in which the transformer (tra) and Sex-lethal genes (SxI) are the main upstream regulators, and the XSEs (X-linked signalling elements) are the primary signal for sex determination (Erickson and Quintero, 2007). This sex determining regulatory cascade (XSE> Sxl> tra> dsx) is based mainly on sex-specific alternative splicing: the protein product of a gene controls the sex-specific splicing of the pre-mRNA produced from a downstream gene in the genetic cascade (Nagoshi et al., 1988). SxI plays the key role of maintaining female sex determination by a positive autoregulation (Cline, 1984). The transformer gene occupies an intermediate position in this hierarchy and is necessary for all aspects of female somatic sexual differentiation. The dsx gene regulates most aspects of somatic sexual differentiation and encodes two protein isoforms, DSX^M and DSX^F, through sex-specific splicing of its primary transcript (Baker and Wolfner, 1988). The processing of the dsx pre-mRNA in females requires the activity of the tragen gene and of transformer-2 gene (tra-2), which encodes a non-sex-specific auxiliary factor with similarity to the SR family of RNA-binding proteins (Amrein et al., 1988, Hoshijima et al., 1991). The two DSX isoforms are responsible to promote male and female sexual development, respectively, by activating or repressing the transcription of a series of target genes in the two sexes (Burtis and Baker, 1989).

The doublesex gene is known to act in concert with other regulatory genes to control the development of the sexual dimorphic structures. For instance, in the Drosophila genital disc, which gives rise to the terminalia and which is composed of two genital primordia (Sanchez and Guerrero, 2001), dsx acts together with the homeotic gene Abdominal-B (Abd-B) to determine which of the two genital primordia will develop and which will be repressed (Keisman and Baker, 2001, Sanchez and Guerrero, 2001). The bric-a-brac gene (bat) is involved in the sexually dimorphic pigmentation of the 5th and 6th abdominal tergites by integrating inputs from dsx and Abd-B (Kopp et al., 2000). Another integrated genetic input made up by dsx and the homoeotic gene Sex combs reduced (Scr) has been invoked for sex-specific differentiation of the basitarsus of the prothoracic leg, developing sex combs in males (Jursnich and Burtis, 1993).

We have previously reported that similar to Drosophila, the Ceratitis capitata transformer homologue (Cctra) is required for the female-specific splicing of the Ceratitis dsx pre-mRNA (Graham et al., 2003, Pane et al., 2002). The evolutionary conservation of the tra-dsx genetic module was recently revealed also in other related Tephritidae as Bactrocera oleae (Lagos et al., 2007) and various Anastrephas species (Ruiz et al., 2007b). The molecular mechanism of dsx regulation seems to be fairly conserved in insects since tra-2 homologues have been identified in Musca domestica (Burghardt et al., 2005) and in Ceratitis capitata (Salvemini et al., submitted) and are required in both these...
species for female-specific dsx splicing.

This study reports the structure, the regulation and the evolutionary functional analysis of the *Ceratitis dsx* orthologue (*Ccdsx*), performed by expressing the male-specific *Ccdsx* isoform in transgenic *Drosophila* flies.

**Results**

To isolate the *Ceratitis dsx* orthologue a radioactive probe was prepared from the *Drosophila* female-specific dsx cDNA, which is highly conserved in other Tephritidae species, such as *Bactrocera tryoni* (Shearman and Frommer, 1998) and *Bactrocera oleae* (Lagos et al., 2005, Shearman and Frommer, 1998). The probe was used to screen two *Ceratitis* cDNA libraries (prepared respectively from female adults and dissected ovaries). Two clones, named F1 (1.1 Kb, from female adults) and Ov1 (1.6 Kb, from ovaries), were isolated and sequenced. Their sequences match a putative dsx ORF truncated in the amino-terminus. RT-PCR analysis on sexed adult flies confirmed that the two cDNA clones correspond to part of female-specific *Ccdsx* mRNAs. A PCR-based screening of a genomic medfly library led to the isolation of two additional non overlapping clones corresponding to the 5’ and internal regions of the gene. RT-PCR analyses were performed to isolate the full-length *Ccdsx* ORFs encoding the CcDSXM and CcDSX F proteins. A 1.3 Kb male-specific cDNA fragment (M1) was amplified by using the BtdsxM reversed primer (designed on the *Bactrocera oleae* dsx male-specific

![Fig. 1. Comparison of the DSX predicted polypeptides in Dipteran species.](image)

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<th>Species</th>
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<td><em>Drosophila melanogaster</em> (Dm) (Burris and Baker, 1989), <em>Ceratitis capitata</em> (Cc) (this work), <em>Anastrepha obliqua</em> (Ao) (Ruiz et al., 2007a, Ruiz et al., 2005), <em>Bactrocera oleae</em> (Bo) (Lagos et al., 2005), <em>Bactrocera tryoni</em> (Bt) (Shearman and Frommer, 1998) and <em>Musca domestica</em> (housefly) (Md) (Hediger et al., 2004).</td>
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faces for protein-protein and protein-DNA interactions (An et al., 1996, Cho and Wensink, 1997). The Ccdsx sex-specific transcripts share a common region composed of two exons, but differ for the presence of a female- and a male-specific exons (Fig. 2). The exon/intron junctions depicted in the schematic in Fig. 2B have been unambiguously determined, comparing sex-specific cDNA and genomic sequences. Nucleotide sequence analysis of C. capitata dsx led to identify sequence conservation of two cis regulatory elements described in the Drosophila dsx gene: 1) a weak polypyrimidine tract at the 3’ acceptor splice site before the female-specific exon and 2) dsxRE elements (putative Tra/Tra-2 binding sites) in the female-specific 3’ untranslated region (Fig. 3).

The timing and the sex-specific pattern of expression of Ccdsx was studied by northern blot and RT-PCR analyses. Northern blot on total RNA, extracted from adult males and females, revealed that a prominent 3.6 Kb long transcript is present in males (Probe C, derived from 5’ end of the Ov1 clone; Fig. 4A) while a single 3 Kb long transcript is produced in females (identified by Probe C and Probe F, derived from 3’ end of the Ov1 clone; Fig. 4A; the weaker signal by the probe F is due to different exposure time). RT-PCR experiments were performed with an oligonucleotide mix containing one single forward (1400+) and 2 reverse primers (the male-specific M1130- and the female-specific F2000-) on total RNA extracted from unfertilized eggs, embryos, larvae and pupae as well as from sexed adult flies. A single 0.6 Kb long cDNA product was observed in unfertilized eggs, embryos, larvae and pupae as well as from sexed adult flies. A single 0.6 Kb long cDNA product was observed in unfertilized eggs, embryos, larvae and pupae as well as from sexed adult flies.
regions, and no evident reduction in viability or fertility. However, females of all transgenic lines showed full male-like pigmentation of the sixth tergite and variable pigmentation also of the fifth tergite (Fig. 5A – as in dsxM12 line). More severe adult phenotypes, similar to those described by Jursnich and Burtis (1993), were observed when transgenic animals were reared at 29°C and exposed to two daily heat shock regimens, from first instar larvae till adult stages: 1) transformation of legs bristles towards a sex-combs-like morphology (Fig. 5B), and larval/pupal lethality, with only a low number of adult escapers (approx 70-90% lethality). Masculinized females showed 2) full male-like pigmentation of the fifth tergite (Fig. 5A – as in dsxM6 line), and 3) transformed genitalia, with reduction in the size of the vaginal plates and in the number of vaginal teeth (Fig. 5C). However, no pigmentation of dorsal spinules and ventral setae were observed in third-instar transgenic larvae. In two different transgenic lines, recovered after heat shock treatment, a few pseudomales (12 out of 70 survivors) showed strongly altered (7 flies) or even almost completely masculinized (5 flies) genitalia. Upon dissection these pseudomales displayed defective female or male germline tissues (Fig 5D). PCR-based analysis was used to assess the karyo-

**Fig. 3 (Above).** Distribution of Tra/Tra-2 binding sites (dsxRE elements) and Purine Rich Element (PRE) in the 3′ untranslated region of dsx female-specific exons. (A) D. melanogaster and (B) C. capitata. The dsxRE are marked in grey boxes. The PRE is marked as a lined box and the respective putative sequence is reported below. Putative polyadenylation signals are marked as black boxes and the stop codons are indicated. On the right of each diagram the dsxRE sequences present in the female-specific exon of the respective species are shown. The shading indicates identical nucleotides.

**Fig. 4.** Timing of expression of the C. capitata dsx gene. (A) Northern blots of total RNA from adult males (XY) and females (XX). Northerns were hybridized with two DNA probes obtained from the Ccdsx female cDNA: probe C, corresponding to the common region of the Ccdsx gene, and probe F, corresponding to female-specific region. (B) RT-PCR analyses on total RNA of unfertilized eggs (UE), embryos 3-20 h old (E), larvae (L), pupae (P), male (XY) and female (XX) adults. At the bottom of (B), the molecular organization of the corresponding cDNAs and the localization of RT-PCR primers (red arrows) are shown.
type of pseudomales from lines 2 and 11 using two Y-specific primers and, as positive internal control, two primers specific for the autosomic \textit{Dmdsx} gene (Fig. 5E). The lack of Y-specific amplification in pseudomales samples confirm their XX karyotype.

**Discussion**

In this study we report the isolation and characterization of the \textit{Ceratitis capitata dsx} gene (\textit{Ccdsx}). As in \textit{Drosophila}, \textit{Musca} and in two Tephritidae species, the \textit{Ccdsx} gene is transcribed in males and females individuals, from early stages of development till adulthood, and its primary transcript undergoes sex-specific splicing, producing the female \textit{CcDSXF} and male \textit{CcDSX}M proteins. The presence of conserved Tra/Tra-2 binding sites within the female-specific exon of \textit{Ceratitis capitata dsx} gene further suggests that also in this species the \textit{dsx} female-specific splicing may be regulated by the gene products of \textit{Cctra} and \textit{Cctra-2}. Hence \textit{Ceratitis dsx} female-specific splicing appears to be regulated by a conserved alternative splicing mechanism in which, as in \textit{Drosophila}, during development the male-specific mode is the default state (default: absence of \textit{CcTRA}), while the female-specific mode is the regulated state, which requires the positive activity of the \textit{CcTRA}/\textit{CcTRA-2} splicing complex, as also suggested by in vivo RNAi against \textit{Cctra} and \textit{Cctra-2} (Pane \textit{et al.}, 2002; Salvemini \textit{et al.}, submitted). While in unfertilized eggs and XX/XY embryos (3-20h) only the \textit{Ccdsx} female-specific variant is amplified by RT-PCR, the male-specific variant seems to be present later on, from early larval stages. Hence we propose that \textit{Ceratitis} male-sex differentiation is controlled by \textit{CcdsxM} as \textit{Dm dsxM} in \textit{Drosophila}, and it starts from larval developmental stages. It is presently thinkable that at embryonal stages sex determination of \textit{Ceratitis} is set up by default in a female mode as suggested by the presence of maternal mRNAs corresponding to \textit{CcdsxF}, but also \textit{Cctra} and \textit{Cctra-2} (Salvemini \textit{et al.}, submitted) and that later on only in XY embryos \textit{Cctra} and consequently \textit{Ccdsx} switch the splicing pattern to the male mode, because of the action of the Y-linked male determining factor, still to be molecularly identified.

We presented data showing that the male-specific \textit{CcDSX}M isoform induces a strong masculinization when expressed in \textit{Drosophila} transgenic females. These results indicate that the \textit{CcDSX}M protein can efficiently outweigh the endogenous \textit{DmDSXF} protein causing a masculinization of the sexual dimorphic structures of the XX transgenic flies. These results further show the biochemical capacity of the \textit{CcDSX}M protein to interact with the
other regulatory partners of the *Drosophila* DSX protein and support the idea of its functional conservation in *Ceratitidis* sex determination. Interestingly, in the case of *tra* which is a functional conserved key female-determining gene in both *Ceratitidis* and *Drosophila*, Pane et al. (2005) showed that CcTRA protein, although very weakly conserved in its sequence (18% identity) and length (Cc 429 aa versus Dm 197 aa) is able to efficiently feminize *Drosophila* XY transgenic flies. On the contrary in the case of Sxl which is a key female-determining gene in *Drosophila* but not in *Ceratitidis*, Saccone et al. (1998) showed that the CcSXL protein, although very highly conserved is not able to efficiently feminize or kill (acting on dosage compensation) *Drosophila* XY transgenic flies, as expected in the case of conservation of its biochemical properties. These two previous studies in which an heterologous functional test for *Ceratitidis* genes was successfully used, support the potential consistency of this “indirect” approach to evolutionary genetic functional conservation. Hence we suggest that not only the biochemical but also the developmental roles of the dsxM homologues are similar in both *Ceratitidis* and *Drosophila*.

A similar experiment using *Musca domestica* DSXM transgene, driven by UAS/hsp70-GAL4 system (which usually permit higher expression levels with the respect of the simple hsp70 fusion construct) in *Drosophila* lines, led only to male-like pigmentation of the posterior tergites (Hediger et al., 2004). The extent of pigmentation was much less pronounced than the one observed with CcDSXM, possibly because the higher sequence similarity of CcDSXM to DmDSXM (58% Cc/Dm versus 50% Md/Dm), and the closer phylogenetic relationship of *Ceratitidis* to *Drosophila* (both belong to Acalyptratae), than to *Musca* (Calyptratae).

It has been shown that the sex of *Drosophila* germ line requires cell-autonomous and inductive signals from the gonadal soma (Hempel and Oliver, 2007, Nothiger et al. (Hempel and Oliver, 2007, Nothiger). The screening of *Drosophila* dsx gene was successful only when the two regulatory partners of the *doublesex* transcription factors specific oligomerization domains in both of the *doublesex* transcription factors were sub-cloned in pUC18 cloning vector (Promega).

**Northern blots and RT-PCR**

Northern blots and hybridizations were performed according to standard protocols (Maniatis et al., 1982). For RT-PCR analyses two micrograms of total RNA from each developmental stage were reverse transcribed with the Superscript II RNase H – reverse transcriptase (Invitrogen) using oligo dT primers and following the manufacturer’s instructions. 1/20 v/v of the synthesised cDNA was amplified by PCR. RT-PCR products were analyzed by agarose gel electrophoresis. The primers used were: CcdsxM 1400+ located in Ccdsx common exon 3, CcdsxM 1130– located in Ccdsx male-specific exon 5 and CcdsxM 2000– located in Ccdsx female-specific exon 4. RT-PCR products were gel-purified, cloned using the pGEM-T Easy Vector Kit (Promega) and sequenced with Big Dye® Terminator v.1.1 Sequencing Kit (Applied Biosystem).

**Generation of CcDSXM Drosophila transgenic lines**

Germline transformation experiments were performed as described by Rubin and Spradling (Rubin and Spradling, 1982), Preblastoderm embryos of *D. melanogaster* were injected with 500 ng/µl of pCaSpeR-hs-Ccdsx act-vector and 150 ng/µl of the P-element A2-3 helper vector and 20 transgenic lines were produced. To induce the expression of the Ccdsx DNA from the Hsp70 promoter, flies from each line were reared at 29°C and exposed twice a day to heat-shock regimen at 37°C for 1 hr, from embryonic stages until adulthood.

**Karyotyping of Drosophila pseudo-females**

Genomic DNA of pseudo-females from transgenic lines 2 and 11 was extracted using the *Drosophila* DNA/RNA Isolation protocol developed by Andres and Thummel (Andres and Thummel, 1994). Y-specific PCR was performed for each line on 50 ng of the extracted genomic DNA with 1 unit of Taq Polymerase (Amersham Pharmacia) according to the manufacturer’s directions in the presence of the specific primers for DmCCY gene and of Dmdsx gene as internal control (CCY+, CCY-, DMF and DF primers).

**Sequences of primers utilized in this paper**

| Dmdsx2251+ | 5'-AGCCATTTCGGCCGTCCTC-3' |
| Dmdsx2541- | 5'-TCATTCCACATTGCCGGG-3' |
| BtdsxM | 5'-TGTTGCTGTAGTAGCTGC-3' |
| CcdsxP+ | 5'-ATAGGAATGGATGAGCTCTT-3' |
| CcdsxC 14400+ | 5'-GGCATCAAGGGTATAGAAGA-3' |
| CcdsxM 1130– | 5'-GCGTTGTGCGATCGATCTCG-3' |
| CcdsxF 2000– | 5'-AGGATGCACTTACCGTAT-3' |
| CCY+ | 5'-CAGTGAAGTCGGTCTC-3' |
| CCY- | 5'-ATTGGTACCAGTACGT-3' |
| DMF | 5'-CCGTATCTTGGAGCT-3' |
| DF | 5'-TTGAGATTTGGTTCTATGCC-3' |

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**References**


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