Genetics and genomics of *Tribolium* Medea elements

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Abstract

Tribolium castaneum, the red flour beetle, is the genetic model for coleopteran insects and the first to have a sequenced genome. It also harbors a fascinating class of selfish genetic elements known as *Maternal-Effect Dominant Embryonic Arrest* (*Medea*) factors. They are widespread in natural populations of Tribolium, but prior to their discovery, were completely unknown in the invertebrate world. *Medea* factors are of considerable interest because each *Medea* allele appears to be bifunctional, encoding both a maternally loaded "toxin" and a zygotically-expressed "antidote". Therefore, only progeny carrying a copy of the *Medea* gene survive. This makes *Medea* a promising candidate for driving anti-pathogen genes into populations of disease-carrying insects. However, despite having determined that the maternal-lethal activity of a *Medea* factor (M^1) is associated with a 21.5-kb insertion, the molecular mechanism by which this selfish element circumvents Mendelian inheritance is still unknown. A major roadblock has been the fact that the sequence within the 21.5-kb insertion is repetitive. Specifically, the sequence contains incomplete or defective copies of genes whose functional copies and non-functional counterparts are found elsewhere in the genome. The one potentially functional gene is most closely related to a group of bacterial genes of unknown function, but is surprisingly found throughout the wild-type Tribolium genome. In an effort to reveal the identity of the maternally loaded toxin we have been performing RNA-Seq on unfertilized eggs obtained from virgin *Medea* females, and comparing the *Medea* egg transcriptome to that from unfertilized wild-type eggs. We are also in the process of performing RNA-Seq on "doomed" wild-type embryos from a heterozygous M^1 female crossed to a wild-type male. We have been mining these data, paying particular attention to sequences which may have originated from the 21.5-kb insertion.

Keywords: *Medea*, red flour beetle, selfish gene, *Tribolium*

1. *Tribolium* as a genetic model

The red flour beetle, *Tribolium castaneum*, is a cosmopolitan pest of stored grains. However, despite its pest status, *T. castaneum* has emerged as a sophisticated model system for the study of evolutionary developmental biology and comparative genomics (Brown et al., 2009). This small, low-maintenance beetle has a relatively short generation time – approximately 4 weeks from egg to adult at 30°C – and is exceedingly easy to rear in the lab. Moreover, Tribolium has a completed and annotated genome sequence (Tribolium Genome Consortium, 2008), as well as a wide range of genetic tools for genome manipulation, including; germline transformation (Lorenzen et al., 2003), transposon-mediated insertional mutagenesis (Lorenzen et al., 2007; Trauner et al., 2009), systemic RNA interference (Brown et al., 1998; Arakane et al., 2005) and efforts are currently underway to employ CRISPR-mediated genome-editing.

Flour beetles (mainly *T. castaneum* and *T. confusum*) first gained prominence as genetic models in the late 1950s and information from those studies can be found in Alexander

Sokoloff's series of books on Tribolium biology (1966, 1972, 1974, 1976), as well as a series of bulletins he published between 1958 and 2002 (http://spiru.cgahr.ksu.edu/proj/tib/). The second wave of *Tribolium* genetics began in the late 1980s with the discovery of over 50 homeotic mutations in Tribolium (see Beeman et al., 1989). This finding came at a time of rapid advancement in molecular biology.

One of the most powerful advances was the use of the P-transposable element for germline transformation in *Drosophila melanogaster*. This technique allowed researchers to add genes to the Drosophila genome (Spradling and Rubin, 1983), and depending on where the P element integrated into the genome, the insertion could knock-out gene function, thus creating new mutant strains. Unfortunately, P-element function is restricted to Drosophilids (Handler et al., 1993), so researchers working on non-Drosophilid species were forced to find alternatives – transposons that would function in their species of interest.

P-elements were discovered as a result of crossing wild-caught Drosophila flies with those from laboratory stocks. Progeny from such crosses had exceptionally high mutation rates, as well as increases in sterility, male recombination, nondisjunction and transmission ratio distortion (Kidwell et al., 1977). However, this phenotype (hybrid dysgenesis) only occurred when wild-caught males were crossed to lab-reared females. The cause was mobilization of P-transposable elements inherited from the male. Interestingly, P-element bearing females express a Piwi-interacting RNA that apparently silences P transposase, the enzyme required for remobilization of the P transposon (Brennecke et al., 2008).

In an effort to discover active transposons in Tribolium, Richard Beeman, a USDA researcher in Manhattan, KS, requested Tribolium samples from around the world. These wild-caught beetles were crossed to a laboratory strain, *GA-1* (Haliscak and Beeman, 1983), which originated in North America. Although these crosses never showed signs of hybrid dysgenesis (i.e. increased rates of sterility and/or mutation), they did reveal a new phenotype. Specifically, when F1 females from a cross between *GA-1* and *SP* (a strain from Singapore) were backcrossed to a *GA-1* male, no *GA-1* progeny survived (Beeman et al., 1992). What Beeman and co-workers discovered was a new type of selfish genetic element. Due to the observed maternal-effect lethality, these elements were named *Medea*, which stands for *Maternal-Effect Dominant Embryonic Arrest*.

2. Genetics and genomics of Medea

Though similar maternal-effect phenomena have been identified in mice (Hurst, 1993; Peters and Barker, 1993), *T. castaneum* and its sister species, *T. confusum*, are the only invertebrate species in which the *Medea* syndrome has been observed and described (Beeman et al., 1992, Wade and Beeman, 1994; Beeman and Friesen, 1999). Each of the four known *Medea* elements (M^{1-4}) is hypothesized to combine a maternal poison and a zygotic antidote to gain a postzygotic survival advantage. Heterozygous ($M/+$) females transmit dominant-lethal activity to hatchlings by maternal action, but the lethal effect is manifested only in those progeny that fail to inherit an *M* allele from either parent (Fig. 1 and Beeman et al., 1992). M^1 and M^4, which are the two most prevalent *M* factors in wild populations, have independent mechanisms of maternal lethality and zygotic rescue. Thus, a zygotic M^4 allele protects the zygote only from maternal M^4, but gives no protection from maternal M^1, and *vice versa*.

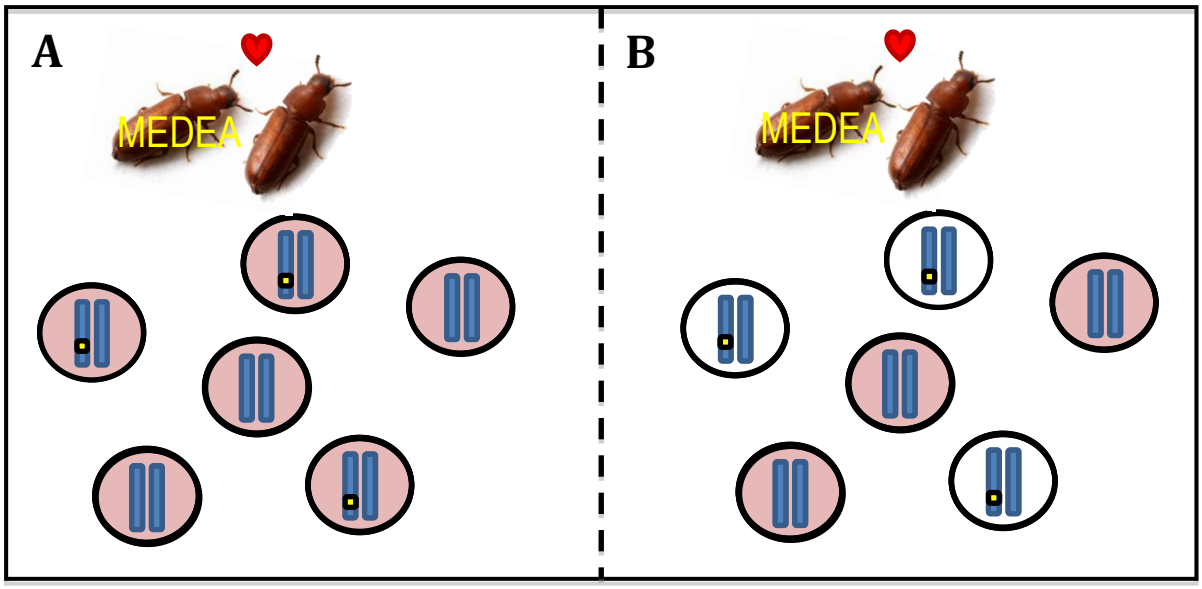

Figure 1 Hypothesized mechanism of *Medea* maternal-effect lethality. The simplest explanation put forth to date is that *Medea* females preload a toxin into every egg. Eggs/embryos are depicted as circles, while chromosomes are shown as blue bars. Pink fill represents toxin and the small yellow boxes denote a copy of the *Medea* gene. A) Early embryos from a heterozygous female mated to a wild-type male. B) Embryos after initiation of zygotic expression. Note that all embryos inheriting the *Medea* gene express the antidote, thus detoxifying (clearing) the maternally-loaded toxin.

Prior to the completion of the Tribolium genome sequence we recombinationally mapped the M^1 locus at a very high resolution of 2 kb, enabling us to positionally clone the region and to identify the M^1 lesion. We discovered that the maternal-lethal activity of M^1 is associated with insertion of a 21.5-kb composite transposon of the *Tc1/mariner* superfamily. The insertion is located between two neuronal genes (Fig. 2 and Lorenzen et al., 2008). Like *Medea* itself, one of these genes, the ortholog of the Drosophila neurotransmitter reuptake symporter *bloated tubules* (*blot*), has both maternal and zygotic functions, at least in Drosophila (Johnson et al., 1999). The other gene is the ortholog of Drosophila *highwire* (*hiw*, Wan et al., 2000). In Drosophila, Highwire negatively regulates synaptic proliferation at neuromuscular junctions through interaction with the Drosophila BMP signaling cascade.

To demonstrate that the 21.5-kb insertion was correlated with the selfish *Medea* allele, we examined wild strains of Tribolium that either possessed or lacked the M^1 activity, collected across various countries and continents. Each strain was retested genetically to confirm the presence or absence of the maternal-lethal M^1 allele, and also tested molecularly for the presence or absence of the insertion. DNA sequencing revealed that about half of these strains were homozygous for the M^1 allele, while the other half were homozygous for the non-*M* (wild-type) allele at the M^1 locus. A perfect correlation was observed (Lorenzen et al., 2008), consistent with the idea that the 21.5-kb insertion is causally associated with maternal-lethal activity.

The 21.5-kb insertion comprises a large, composite *Tc1* transposon. The insertion itself contains incomplete or defective copies of at least three genes, namely *ATP synthase subunit C* (*ATPsyntC*), *elongation initiation factor 3* (*eIF3*), and a gene of unknown function

encoding a helicase RNaseD C-terminal domain (HRDC). Orthologs of *ATPsyntC* and *eIF3* are vital in Drosophila, and intact copies occur elsewhere in the Tribolium genome (the fully sequenced, non-*M* strain). In addition to these genes, there is a fourth gene, DUF1703 (domain of unknown function 1703, aka protein of unknown function 1703), having similarity mainly to genes from bacteria.

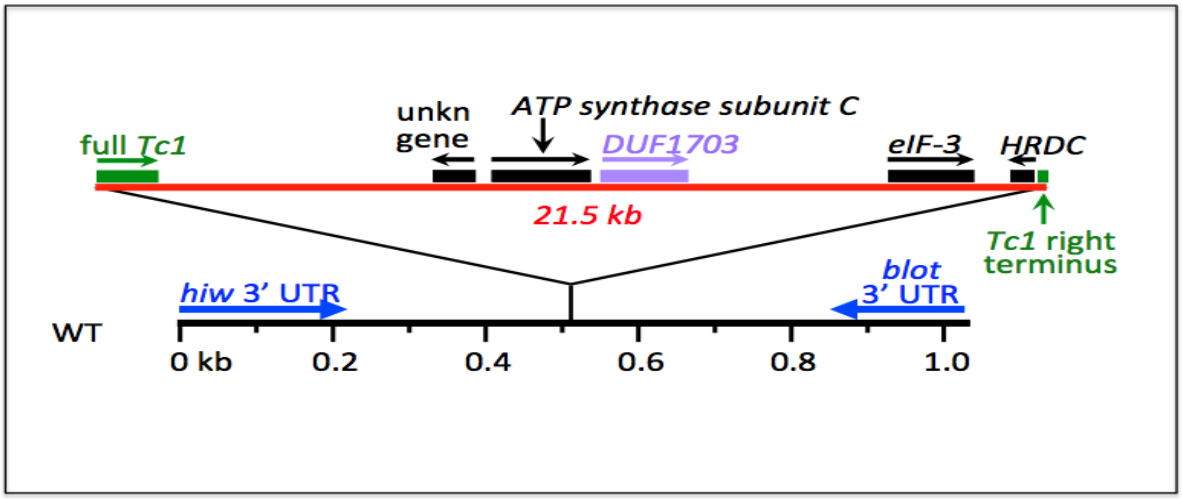

Figure 2 Insertion of a 21.5-kb Tc1/mariner transposon in the M^1 strain of *T. castaneum*. The segment is inserted near the middle of the 800-nt region separating the 3' ends of the Tribolium orthologs of the Drosophila genes, *highwire* and *blot*. Arrows indicate directions of transcription, or orientations of repeats. Tc1/mariner full sequence and isolated inverted terminal repeat flanking the insert are shown in green. Not drawn to scale.

DUF1703 is a member of Pfam08011, which includes restriction endonucleases, transposases, recombinases, and a variety of proteins thought to perhaps protect DNA from nuclease action. Of the 1,360 DUF1703 proteins currently recognized by EMBL-EBI, 1,334 are from bacteria, while only two are from arthropods (both from Tribolium). Interestingly, sequence analysis indicates that DUF1703 is the only potentially functional gene present in the *Tc1* insertion. However, there are at least 26 DUF1703-related genes in the wild-type Tribolium genome. Therefore, in spite of our characterization of the M^1 region, the molecular mechanisms of M^1 maternal lethality and zygotic protection are not obvious.

Functional studies of M^1 have, however, continued to focus on characterizing the DUF1703 genes, since they may have arisen from an ancient lateral gene transfer event. Phylogenetic analysis indicates that the DUF1703-like genes have not only persisted in the beetle genome, they have undergone expansion. There are at least 26 DUF1703 family members scattered throughout the genome, encompassing at least seven subfamilies; the degree of conservation between subfamily members suggests that many of these genes are functional.

In an effort to understand what role DUF1703 genes play in Tribolium we used RNA interference (RNAi) to reduce DUF1703 expression. However, sequence identity within each subfamily is high enough that dsRNA targeting one subfamily member, results in RNAi-mediated knockdown of all members of the targeted subfamily. Although RNAi targeting subfamilies-2 through 7 had no obvious effect, subfamily-1 RNAi was found to be lethal in both wild type and M^1 individuals (Fig. 3). This suggests that at least one subfamily-1

member has taken on an essential role in Tribolium development. It is, of course, possible that death is the result of an additive effect, due to the loss of multiple subfamily-1 gene members, rather than the loss of any single member alone, but this does not diminish the possibility of an essential role.

Regardless of the life stage tested (embryo, larva, pupae or adult), injected individuals appear to discontinue or postpone development and eventually waste away. For example, depending on the timing of injection, some last-instar larvae stop feeding, cease development and slowly waste away (Fig. 3 B & C), while others pupate, but immediately afterward cease development and waste away (Fig. 3 D & E). One obvious phenotype (other than death) is the conspicuous loss of uniformity in the ommatidia. Neither class of subfamily-1 dsRNA-injected larvae ever reaches adulthood despite length of survival (i.e. buffer-injected individuals enclose ~5 days post injection, while dsRNA-injected individuals remain alive, but either arrest as larvae, or as pupae. To gain a better understanding of these genes we are in the process of deleting the M^1 version of DUF1703 using the CRISPR/Cas9 system (for a review on CRISPR/Cas9 see Peng et al., 2014).

Figure 3 Effect of sub-1 RNAi on last-instar larvae. (A) buffer injected larva 12-days post treatment, and RT-PCR of RPS6 control vs. sub1 demonstrating concomitant expression levels. (B) sub-1 dsRNA injected larva 12-days post injection. RT-PCR indicates knockout of gene sub1 transcript. (C) dsRNA injected 22-days post injection. (D) buffer injected larva after pupation. (E) dsRNA injected larva after pupation (note abnormal ommatidia development; white arrows).

It seems unlikely that DUF1703 plays a role in *Medea* since there are around 26 copies in the wild-type genome. Therefore, we are using RNA-Seq to compare transcripts found in unfertilized eggs from M^1 and *GA-1* females in an attempt to identify the "toxin" responsible for M^1-mediated lethality. However, it is also possible that the sequence within the 21.5-kb region is altering global gene expression. Therefore, to address this question we are performing RNA-Seq on unfertilized eggs (M^1 vs. non-*M*), embryos (M^1/+ vs. +/+; where +/+ are doomed due to M^1 lethality), and newly hatched larvae (also from a cross giving 50% doomed offspring). Preliminary results suggest significant differences in gene regulation, and point to the possibility of an epigenetic effect. For example, we see 2x upregulation of *histone deactylase 8* in M^1 eggs. Histone deacetylation provides a tag for epigenetic repression and is known to play an important role in transcriptional regulation. Therefore it is possible that

Medea toxicity is due to drastically different transcriptional states between chromosomes inherited from *Medea* females and wild-type males.

3. Conclusions

While *Medea* elements are of considerable interest from the viewpoint of basic evolutionary and population biology, they are also of interest due to their potential application as gene drivers that could enable the genetic control of vector-borne diseases, decrease crop losses to pest insects and rodents, and protect endangered species (James, 2005; Gould et al., 2006; Gould, 2008). Indeed the motivation for the development of a synthetic *Medea* element in Drosophila by Chen et al., (2007) was as proof of principle that similar *Medea* elements could be engineered into mosquitoes that transmit Dengue virus and the *Plasmodium* that causes human malaria. Discovering the function of the naturally-occurring *Medea* elements would undoubtedly aid efforts in creating reliable gene drive systems for other species.

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