RNA INTERFERENCE IN THE RED FLOUR BEETLE *TRIBOLIUM CASTANEUM*

by

SHERRY C. MILLER

B.S., Truman State University, Kirksville, Missouri USA, 2003

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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ABSTRACT

RNA interference (RNAi) is a natural gene-silencing phenomenon triggered by dsRNA (dsRNA). While RNAi is an endogenous process that plays essential roles in regulating gene expression it can also be harnessed as a tool for the study of gene function. Introducing dsRNA that is homologous to target mRNA into a cell triggers the RNAi response causing the destruction of the homologous mRNA and a loss of function phenotype. In some organisms, such as the nematode *Caenorhabditis elegans*, once dsRNA is introduced into the body cavity, the RNAi effect is seen throughout the organism because the dsRNA is taken up by individual cells and is then spread from cell to cell. This process has been termed the systemic RNAi response. For other organisms, such as the fruit fly *Drosophila melanogaster*, introduction of dsRNA into the body cavity does not result in a systemic RNAi response. This may be due to the cell's inability to take up dsRNA or spread that dsRNA from cell to cell. For other organisms, including mammals, introduction of dsRNA into the body cavity does not result in a systemic RNAi response because the immune response causes dsRNA destruction before it can be utilized in the RNAi pathway. For organisms that do not exhibit a systemic RNAi response, complex genetic methods are needed to introduce dsRNA into cells to induce the RNAi response. Therefore, one of the challenges in utilizing RNAi as a genetic tool is introducing the dsRNA into individual cells.

In recent years, systemic RNAi responses have been documented in both model and non-model organisms, making RNAi an accessible genetic tool. The red flour beetle, *Tribolium castaneum* is an emerging model organism that has a robust systemic RNAi response. However, the mechanism of systemic RNAi and the specific parameters

required to obtain a strong systemic RNAi response in this organism have not been thoroughly investigated. The aim of this work is to provide data that can allow RNAi to be better utilized as a genetic tool in *Tribolium* and to use this information as a basis for the use of RNAi in other insects in which it can be performed. Specifically we provide data on the essential parameters necessary to achieve an effective systemic response in *Tribolium*, we describe differences in the systemic RNAi response between *Drosophila* and *Tribolium*, we analyze the conservation and function of RNAi machinery genes in *Tribolium* and we provide information on the genes critical for a systemic RNAi response in *Tribolium*.

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Introduction

RNAi

The RNA interference (RNAi) phenomenon, in which dsRNA induces gene silencing, was first documented in 1990 when it was discovered that the introduction of a chalcone synthase (CHS) transgene into petunia plants resulted in silencing (cosupression) of the endogenous CHS gene (Napoli et al. 1990). Soon after documentation of this "cosupression" phenomenon, it was discovered that homologous RNA sequences could also silence gene function in the fungus *Neurospora crassa* by a process described as "quelling" (Romano and Macino 1992). Several years later, mRNA silencing by sense and antisense RNA was described in the animal model, *Caenorhabditis elegans* (Guo and Kemphues 1995). While all of these processes resulted in gene silencing, their mechanism was unknown and therefore they were not recognized as related processes until 1998 when Fire and Mello described double-stranded RNA (dsRNA) as the trigger for RNAi (Fire et al. 1998) (reviewed in Sen and Blau 2006).

Since the identification of dsRNA as the silencing trigger, a decade of work has resulted in elucidation of the molecular mechanism of the "classic" RNAi pathway (Fig 1A). This pathway is composed of two phases, the initiator phase and the effector phase, which together result in post-transcriptional gene silencing (PTGS) (reviewed in Hammond 2005). The initiator phase begins when the dsRNA trigger is bound, with the help of a dsRBM protein, by the type III endonuclease, Dicer. Dicer then mediates the cleavage of dsRNA into short interfering RNAs (siRNAs) approximately 21bp in length (Bernstein et al. 2001; Knight and Bass 2001; Carmell and Hannon 2004). The effector phase of the pathway begins when the siRNAs are incorporated into the multiprotein RNA-induced silencing complex (RISC) (Hammond et al. 2000; Filipowicz 2005). For this to occur, the unincorporated (passenger) strand must be cleaved from the siRNA

duplex by the RNase H activity of an Argonaute (Ago) protein (Matranga et al. 2005; Rand et al. 2005). If the incorporated strand is the antisense strand, then it guides RISC to its homologous target mRNA where the "slicer" activity of the Ago protein causes nucleolytic destruction of the target mRNA (Tabara et al. 1999; Fagard et al. 2000; Hammond et al. 2001; Song et al. 2004).

In addition to the "classic" mechanism described above, RNAi components have also been shown to play a role in two other processes; (1) post-transcriptional silencing (PTGS) through the action of microRNAs (miRNAs) (Fig 1B) (reviewed in Ouellet et al. 2006; Niwa and Slack 2007) and (2) transcriptional gene silencing (TGS) by chromatin modification (Fig 1C) (reviewed in Lippman and Martienssen 2004). Regardless of whether PTGS or TGS is the mechanism of action, gene silencing by the RNAi pathway involves the production of small RNAs and utilizes Ago family members. However, many differences between these RNAi pathways also exist. Generally, the miRNA pathway results in gene silencing through a PTGS mechanism in the cytoplasm, in which target mRNAs are either translationally repressed or degraded (reviewed in Ouellet et al. 2006; Niwa and Slack 2007). The miRNA pathway begins when miRNA precursors are transcribed from a class of non-coding genes. These precursors are primary transcripts that form imperfect dsRNA hairpin structures (pri-miRNA). pri-miRNAs are processed by the RNase-III-type endonuclease Drosha into pre-miRNAs (Lee et al. 2002b; Lee et al. 2003) that are then exported to the cytoplasm via the nuclear export receptor, Exportin-5 (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004). Once in the cytoplasm, pre-miRNAs are further processed by Dicer to produce mature miRNAs (Hutvagner et al. 2001; Lee et al. 2004). Mature miRNAs are incorporated into miRNA-containing effector complexes (miRNPs) that contain a member of the Ago family (Mourelatos et al. 2002). This complex then binds the 3' UTR of target transcripts resulting in either translational repression (Olsen and Ambros 1999;

Seggerson et al. 2002) or mRNA cleavage and degradation (Yekta et al. 2004; Lim et al. 2005) (reviewed in Ouellet et al. 2006; Niwa and Slack 2007). In contrast, TGS can occur by the production of endo-siRNAs or repeat-associated short interfering RNAs (rasiRNAs) (Aravin et al. 2003) later renamed as PIWI-interacting RNAs (piRNA) (reviewed in Meister and Tuschl 2004; Ghildiyal and Zamore 2009). These small RNAs once bound by members of the Ago family are responsible for chromatin modification in the nucleus, which results in gene silencing (reviewed in Meister and Tuschl 2004; Ghildiyal and Zamore 2009).

The study of RNAi has resulted in the elucidation of a highly conserved, complex method of endogenous gene regulation that is mediated through a variety of RNA-based products (siRNAs, miRNAs, or piRNAs). As these studies progress, the vast importance of this pathway is further revealed. The RNAi pathway is an essential mechanism of protection against viral infections (Waterhouse et al. 2001; Wang et al. 2006) and random insertion of transposable elements (Meister and Tuschl 2004). RNAi is also likely to have major implications in the biological role of heterochromatin and genome maintenance (Lippman and Martienssen 2004). Finally, miRNAs play important roles in development and basic cellular processes, influencing the expression of an estimated 30 percent of all protein coding genes (Ouellet et al. 2006).

RNAi as a tool

While RNAi plays an essential role in endogenous gene expression, it has also been harnessed in many model systems as a powerful tool to obtain loss of function phenotypes. Developmental biology, cellular biology, evolutionary biology and functional genomics have all been dramatically impacted by the ability to quickly examine gene function. In addition to the benefit of using RNAi as a tool for defining the roles of genes in biological processes, there are also many other applications for RNAi including medical therapy for viral infection (Shankar et

al. 2005) or genetic diseases (Shankar et al. 2005), pest management (Baum et al. 2007; Mao et al. 2007) and agricultural manipulation (Siritunga and Sayre 2003; Gavilano et al. 2006; Le et al. 2006). The potential for RNAi as a theraputic tool in the medical field is staggering. RNAi has been used successfully to inhibit viral replication of rotavirus, respiratory syncytial virus, influenza virus, poliovirus, West Nile virus, dengue virus, foot and mouth disease virus, human papillomavirus, herpes simplex virus, hepatitis B virus, hepatitis C virus, hepatitis delta virus, coronavirus, JC virus, and human immunodeficiency virus (Shankar et al. 2005). RNAi may also be useful as a theraputic tool for cancer, since dsRNAs designed against oncogenes inhibit the growth and survival of tumor cells (Shankar et al. 2005). Finally, RNAi shows great promise for treating dominantly-inherited neurodegenerative diseases such as Alzheimer's, Huntington's and spinocerebellar ataxia. Clinical trials for RNAi-based treatment of macular degeneration, which is caused by protein overexpression, have already begun (Shankar et al. 2005). In the agricultural arena, the expression of dsRNA in plants may be used to control pests by targeting essential insect genes (Baum et al. 2007; Mao et al. 2007) and may also be used to reduce naturally occurring plant toxins (Siritunga and Sayre 2003), allergens (Le et al. 2006) or carcinogenic compounds (Gavilano et al. 2006), making plants more agriculturally desirable.

While the use of RNAi in biomedical, biotech and basic biological research holds great promise, for some organisms there is a common challenge when using RNAi as a tool. dsRNA must be inside the cell to initiate the RNAi response. Therefore, the delivery of dsRNA into individual cells of a multicellular organism can be problematic. To circumvent this problem several delivery strategies have been developed. First, virus-mediated methods have been used to deliver dsRNA hairpin constructs into individual cells (Brummelkamp et al. 2002b; Qin et al. 2003; Rubinson et al. 2003; Stewart et al. 2003; Uhlirova et al. 2003). Second, siRNAs have

been chemically modified to allow cellular uptake (Henry et al. 2006; Li and Huang 2006; Mook et al. 2007). Third, transgenic approaches have been developed to express long dsRNA hairpins or short-hairpin dsRNAs within individual cells (Fortier and Belote 2000; Kennerdell and Carthew 2000; Tavernarakis et al. 2000; Piccin et al. 2001; Svoboda et al. 2001; Brummelkamp et al. 2002a; Lee et al. 2002a; McManus et al. 2002; Miyagishi and Taira 2002; Paddison et al. 2002a; Paddison et al. 2002b; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002; Kawasaki and Taira 2003). In advanced model systems such as *Drosophila melanogaster* this type of approach has even led to the production of genome-libraries of RNAi transgenes (Dietzl et al. 2007). Finally, for some organisms it is possible to deliver dsRNA directly by injecting dsRNA into embryos at the one cell stage before cell membranes form (Kennerdell and Carthew 1998; Brown et al. 1999). While all of these methods have been successful, they all have certain disadvantages. Viral-mediated methods of dsRNA delivery and chemically modified siRNAs do not provide continuous expression, so silencing is only transient. Transgenic approaches allow for continuous expression (and therefore sustained silencing), but have only been developed for the most advanced model organisms. Egg injections can only be performed in organisms where the egg develops outside the mother's body. These injections result in gene silencing very early in development making the study of pleiotropic gene function difficult. And finally, egg injection is a difficult, laborious process in which few individuals survive.

Systemic RNAi

Fortunately, in some genetic model organisms the application of RNAi as a tool is less problematic, because the cells have the ability to take up dsRNA from the extracellular environment (and in some cases spread the dsRNA to neighboring cells) (Palauqui et al. 1997; Voinnet and Baulcombe 1997; Fire et al. 1998). This process is called systemic RNAi. This

term was coined when it was discovered that, in *C. elegans,* the RNAi effect could be seen throughout the organism regardless of the site of dsRNA injection (Fire et al. 1998). Later, it was discovered that a systemic RNAi response could also be triggered when *C. elegans* were soaked in a solution of dsRNA (Tabara et al. 1998) or fed transgenic *Escherichia coli* expressing dsRNA (Timmons and Fire 1998).

In *C. elegans*, several genes have been identified that play a role in dsRNA uptake and subsequent spreading, although the most important appears to be the gene encoding a transmembrane protein called SID-1 (Winston et al. 2002). SID-1 is essential for uptake of dsRNA into somatic and germline cells (Winston et al. 2002). It is believed to act as a passive dsRNA channel that shows a preference for long dsRNA molecules (Hunter et al. 2006). Other genes have also been identified as critical for dsRNA uptake in *C. elegans*. *sid-2* is essential for dsRNA uptake from the gut but does not appear to play a role in dsRNA spreading beyond the gut (Winston et al. 2007). The presence of *sid-2* may be essential for allowing the environmental introduction of dsRNA in *Caenorhabditis*, as other *Caenorhabditis* species lack a *sid-2* homolog and are unable to respond to environementally provided dsRNA. Additionally, overexpression of *C. elegans sid-2* in *C. briggsae* and *C. remanei* allows them to become susceptible to externally provided dsRNA (Winston et al. 2007; Felix 2008). Additional genes, *rsd-2*, *rsd-3* and *rsd-6* are all essential for the uptake of dsRNA into germline but not somatic cells in *C. elegans* (Tijsterman et al. 2004).

Systemic RNAi has also been documented in plants, which exhibit both cellular uptake and spreading. However, the mechanisms used in plants (short-range transmission of siRNAs through plamodesmata and longer-range transport of longer dsRNA by the phloem vasculature (Himber et al. 2003)) appear to be quite different from those identified in *C. elegans*.

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Besides *C. elegans* and plants, systemic RNAi has been documented in other organisms, including other nematodes (Felix 2008), flatworms (Sanchez Alvarado and Newmark 1999), crustaceans (Robalino et al. 2005), chelicerates (Aljamali et al. 2003; Narasimhan et al. 2004; Soares et al. 2005; Akiyama-Oda and Oda 2006) and insects (Bucher et al. 2002; Tomoyasu and Denell 2004), although the mechanism is unknown. Unfortunately, this lack of data has often led to unsubstantiated assumptions that all animals with a systemic RNAi response use the mechanism described in *C. elegans*. While the mechanism of systemic RNAi in other organisms is not known, it is clear that the systemic RNAi response is not universal, as the leading insect model, *Drosophila melanogaster,* appears to be unable to mount a systemic RNAi response (Roignant et al. 2003).

RNAi in insects

Insects provide powerful models for the study of gene function. Many of them meet the criteria for a genetic model organism, including small body size, short generation time and large brood sizes. Insects represent an extremely diverse group in regard to both morphology and life history traits, which makes them ideal models for comparative studies in the fields of physiology, evolutionary biology, developmental biology and population biology. Additionally, knowledge of insect biology is crucial if we hope to solve the problems they cause as agricultural pests and vectors of disease.

While *Drosophila* is one of the most powerful genetic models, it displays many highly diverged features and may not possess a particular trait of interest. Therefore, there is a need to study other insect models. The development of RNAi as a tool is vital to the study of emerging model insects as it provides a method to study gene function without the development of complex genetic tools. Since its discovery, systemic RNAi has been reported in many insects

including the mosquitoes *Anopheles gambiae* (Blandin et al. 2002), *Aedes aegypti* (Zhu et al. 2003) and *Culex pipiens* (Sim and Denlinger 2008), the moths *Spodoptera litura* (Rajagopal et al. 2002), *Epiphyas postvittana* (Turner et al. 2006), *Manduca sexta* (Eleftherianos et al. 2007), *Hyalophora cecropia* (Bettencourt et al. 2002), *Bombyx mori* (Tabunoki et al. 2004) and *Helicoverpa armigera* (Mao et al. 2007), the milkweed bug *Oncopeltus fasciatus* (Liu and Kaufman 2004), the triatomine bug *Rhodnius prolixus* (Araujo et al. 2006), the locust *Locusta migratoria manilensis* (He 2006; Wei 2007), the pea aphid *Acyrthosiphon pisum* (Mutti 2006), the honeybee *Apis mellifera* (Amdam et al. 2003), the jewel wasp *Nasonia vitripennis* (Lynch and Desplan 2006), the termite *Reticulitermes flavipes* (Zhou et al. 2006), the cockroaches *Blattella germanica* (Cruz et al. 2006) and *Periplaneta americana* (Marie et al. 2000), the cricket *Gryllus bimaculatus* (Meyering-Vos and Muller 2007), the flies *Bemisia tabaci* (Ghanim et al. 2007), *Sarcophaga peregrine* (Nishikawa and Natori 2001), *Glossina morsitans morsitans* (Lehane et al. 2008; Walshe et al. 2008) and *Lutzomyia longipalpis* (Sant'Anna et al. 2008), and the beetles *Harmonia axyridis* (Niimi 2005), *Protaetia brevitarsis* (Kim et al. 2008), *Diabrotica virgifera* (Baum et al. 2007), *Diabrotica undecimpunctaa howardii* (Baum et al. 2007), *Leptinotarsa decemlineata* (Baum et al. 2007) and *Tribolium castaneum* (Bucher et al. 2002; Tomoyasu and Denell 2004) (Fig 2, STable 1).

Among the winged insects approximately 30 species have been shown to exhibit a systemic RNAi response (STable 1). These species cover a wide phylogenetic range, representing a wide variety of body morphologies and life history traits (Fig 2) suggesting possible conservation of the systemic RNAi response in insects. While most RNAi studies in insects are performed by injection of dsRNA there is also limited published data on the effectiveness of feeding dsRNA. Successful feeding has been documented in several insect

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species from Dictyoptera to Endopterygota (Fig 2) suggesting that feeding may also be a possible mechanism of dsRNA delivery, which may provide a powerful insect pest control technique. While both dsRNA injection and feeding have been documented in a variety of insects, there is anecdotal evidence to suggest that in some insects systemic RNAi is at best inefficient. Interestingly, the orders for which difficulties with systemic RNAi is best known, Diptera and Lepidoptera, cluster together (Fig 2), suggesting a possible loss of the mechanism of dsRNA uptake. However, these orders are also two of the best-studied orders. Therefore, it is possible that these problems are not unique to this particular clade but instead represent independent losses in the insect phylogenetic tree that may be speckled with many more species unable to mount a systemic RNAi response. More comprehensive study is necessary before evolutionary conservation of the systemic RNAi response can be inferred.

While RNAi at multicellular stages has been successful in many insects suggesting a conserved systemic RNAi repsponse, studies have been limited with regard to the number of species examined, the number of genes studied, the variety of tissues affected and the life stages susceptible to RNAi (STable 1). Thus, the full utility and conservation of the systemic RNAi response in these insects is unknown. Furthermore, assumptions about the parameters necessary to achieve an effective RNAi response and the mechanism by which the systemic RNAi response occurs are based on the few organisms in which systemic RNAi has been studied (none of which are insects). Therefore, if we hope to effectively use RNAi as a tool for the study of gene function in non-model insects then the systemic RNAi process needs to be thoroughly studied in insects. Since *Tribolium* mounts an extremely robust systemic RNAi response and since it is an established insect model, we have used *Tribolium* as a model to study systemic RNAi in insects. In the following chapters I will provide data on the essential parameters necessary to achieve an

effective systemic RNAi response in *Tribolium*, describe differences in the systemic RNAi response between *Drosophila* and *Tribolium*, analyze the conservation and function of the RNAi machinery genes in *Tribolium* and provide data on the genes essential for the systemic RNAi response in *Tribolium*.

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Supplemental Table 1: Systemic RNAi in insects

Species	Common Name	Method	Stage	Type	Known Suseptible Tissue	Reference #
Anopheles gambiae	mosquitio	injection	adults	dsRNA	fat bodies, midgut, hemocytes, salivary glands	6, 7, 10, 40
Aedes aegypti	dengue mosquitio	injection	adults	dsRNA	fat bodies, midgut	19, 44
Culex pipiens	northern house mosquitio	injection	adults	dsRNA	ovaries	36
Spodoptera litura	armyworm	injection	5th instar larvae	dsRNA	midgut, germ cells	33
Epiphyas postvittana	light brown apple moth	feeding	3rd instar larvae	dsRNA	gut, antennae	39
Manduca sexta	tobacco hornworm	injection	5th instar larvae	dsRNA	hemocytes	13
Hyalophora cecropia	giant silkmoth	injection	pupae	dsRNA	germ cells	5
Bombyx mori *	silkworm	injection	pupae, 5th instar larvae	dsRNA	CCAP neurons, pheromone-producing cells, silk gland	37
Helicoverpa armigera	cotton bollworm	feeding	3rd instar larvae	dsRNA	midgut	24
Oncopeltus fasciatus	milk weed bug	injection	adults	dsRNA	germ cells	22
Rhodnius prolixus	triatomine bug	injection, feeding	2nd & 4th instar nymphs	dsRNA	salivary glands	3
Locusta migratoria	oriental migratory locust	injection	adults, 5th instar larvae	dsRNA	oocytes, epidermis	17, 42
Acyrthosiphon pisum	pea aphid	injection	adults, 3rd instar larvae	siRNA , dsRNA	salivary glands, gut	18, 28
Apis mellifera	Honeybee	injection, feeding	workers	dsRNA	fat bodies, brain, antennal lobe	2, 14, 32
Nasonia vitripennis	jewel wasp	injection	pupae, adults	dsRNA	germ cells, rectal vesicle	1, 23
Reticulitermes flavipes	eastern subterranean termite	injection, feeding	workers	siRNA	fat bodies, hemolymph	43
Blatella germanica	German cockroach	injection	last instar nymphs	dsRNA	prothoracic gland, epidermis, fat bodies	9
Periplaneta americana	American cockroach	injection	1st instar	dsRNA	hemocytes, sensory neurons, epidermis	25
Gryllus bimaculatus	field cricket	injection, feeding	adults, last instar larvae, 3rd instar nymph	dsRNA	oocytes, midgut, leg tissue	26, 27, 29, 34
Bemisia tabaci	whitefly	injection	adult	dsRNA	midgut, salivary glands, ovaries	15
Sarcophaga peregrina	flesh fly	injection	3rd instar larvae	dsRNA	hemocytes	31
Lutzomyia longipalpis	sand fly	injection	adult	dsRNA	unknown	35
Glossina morsitans	tsetse fly	injection, feeding	adult	dsRNA	fat bodies (only succesful with injection), midgut	21, 41
Drosophila melanogaster *	fruitfly	injection	adults	dsRNA	CNS, midgut, body	11, 12, 16
Harmonia axyridis	Asian lady beetle	injection	last instar larvae	dsRNA	appendages	30
Protaetia brevitarsis	white-spotted flower chafer	injection	larvae	dsRNA	fat body and hemolymph	20
Diabrotica virgifera	western corn rootworm	feeding	larvae	dsRNA	unknown⁺	4
Diabrotica undecimpunctata	southern corn rootworm	feeding	larvae	dsRNA	unknown⁺	4
Leptinotarsa decemlineata	Colorado potato beetle	feeding	larvae	dsdRNA	unknown⁺	4
Tribolium castaneum	red flour beetle	injection	larvae, pupae, adults	dsRNA	many including germ cells, ectoderm, midgut	8, 38

*A few papers have been published in which RNAi at post-embryonic stages have been successful. However, most descriptions suggest that this insect does not have a reliable systemic RNAi response.

⁺ Individual tissues were not tested however, injection of dsRNA for a variety of housekeeping genes caused mortality or larval stunting.

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Figure 1

Mechanisms of RNAi. **(A)** Depiction of the "classic" RNAi pathway in which post-transcriptional gene silencing (PTGS) is mediated through the production of short interfering RNAs (siRNAs) and their association with the RNA- induced silencing complex (RISC) and their mRNA targets. **(B)** Depiction of the miRNA pathway in which PTGS is mediated through the production of microRNAs (miRNAs) which cause mRNA cleavage or translational repression once they bind to the 3'UTR of their target. **(C)** Depiction of transcriptional gene silencing (TGS), which utilizes RNAi component proteins to produce repeat-associated short interfering RNAs (rasiRNAs)/PIWI-interacting RNAs (piRNAs) which are bound by Ago proteins and are then capable of directly binding DNA and preventing transcription.

A

|||||||||||||||

Dicer ↓

|||||||

↓

siRNA TTTTTT

RISC ↓

✂

mRNA |||||||||||||||
RNA cleavage

B

||||||||||○

Dicer ↓

|||||

↓

miRNA TTTTTT

miRNP ↓

✂

mRNA |||||||||||||||
RNA cleavage

or

mRNA |||||||||||
Translational repression

C

|||||||||||||||

↓

|||||

↓

rasiRNA/ piRNA TTTTTT

↓

Me Me

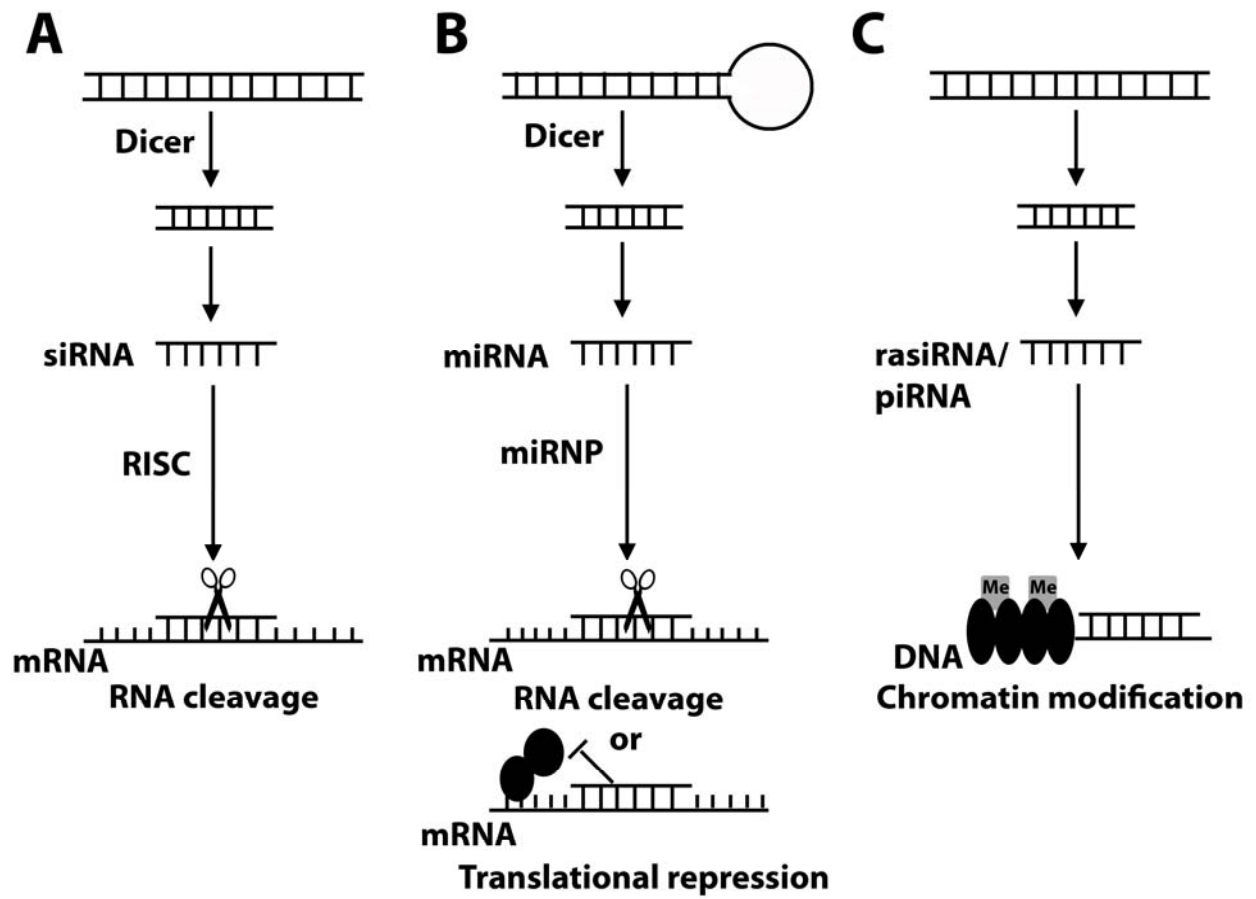**DNA** ●●●● |||||||
Chromatin modification

Figure 2

Phylogenetic tree of winged insects. General tree depicting the relationship between insects capable of mounting a systemic RNAi response. Red and blue text indicataes there have been published reports of a systemic RNAi response in species within these insect orders. Red text indicates that there have also been reports of inefficient or unsuccessful attempts performing RNAi in species within these groups. # species is the number of species for which there is published reports of successful post embryoninc RNAi. + indicates that either siRNA use or dsRNA feeding has been successful. − indicates that siRNAs have been tried and are unsuccessful. For groups without a + or − dsRNA was introduced by injection. ND means there is no published data on the subject. For more detailed information on species, life stage, tissue susceptibility and references see Supplemental Table 1. Please note that the relationships within the holometabolist insects (Endopterygota) are still under debate. While the tree depicted here is the traditional view there is data to suggest that Hymenoptera is more basal than Coleoptera. See (Savard et al. 2006) for more information.

insect order	# species	siRNA	feeding
Ephemeroptera (mayflies)			
Odonata (dragonflies)			
Blattaria (cockroaches)	2	ND	ND
Isoptera (termites)	1	+	+
Orthoptera (crickets and locusts)	2	ND	+/ND
Hemiptera (aphids and true bugs)	3	+/ND	+/ND
Coleoptera (beetles)	6	-/ND	+/ND
Diptera (flies and mosquitoes)	8	ND	+/ND
Lepidoptera (butterflies and moths)	6	ND	ND
Hymenoptera (wasps and bees)	2	ND	+/ND

27
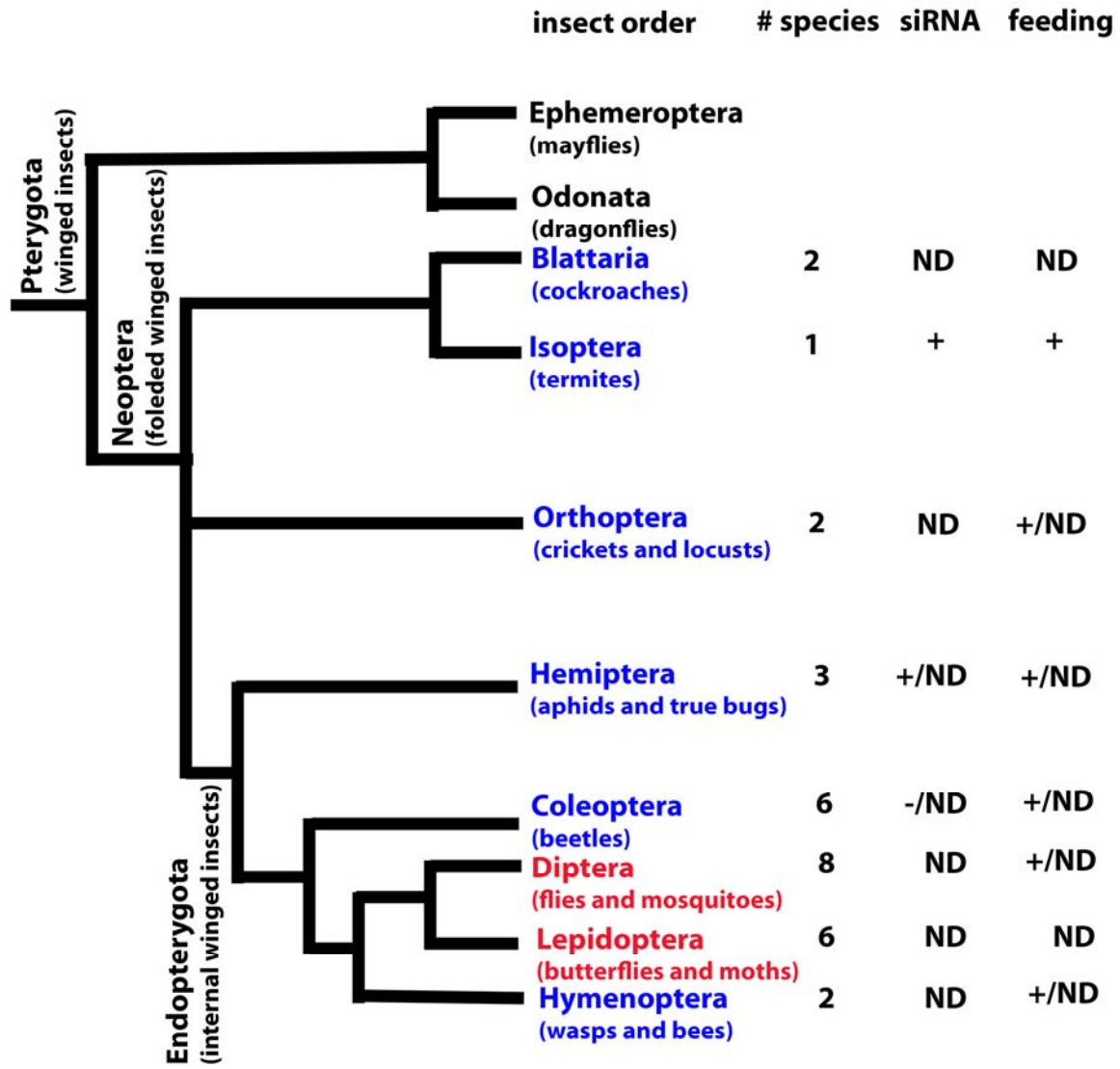
Chapter I

Parameters affecting the success of RNAi in the red flour beetle *Tribolium castaneum*

Running head: RNAi in *Tribolium*

Sherry C. Miller, Susan J. Brown and Yoshinori Tomoyasu*
Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506

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*Corresponding author: Yoshinori Tomoyasu**, Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506
**Current contact information: Department of Zoology, Miami University, 212 Pearson Hall, Oxford, OH 45056, Phone (513) 529-3162, email: tomoyay@muohio.edu

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Abstract

The phenomena of RNAi, in which introduction of dsRNA into a cell triggers the destruction of complementary mRNA resulting in a gene silencing effect, has been shown to be conserved across a wide array of plant and animal phyla. However, the mechanism by which the dsRNA enters a cell allowing the RNAi effect to occur throughout a multicellular organism (systemic RNAi) has only been studied extensively in certain plants and the nematode *Caenorhabditis elegans*. In recent years, RNAi has become a popular reverse genetic technique for gene silencing in both model and non-model systems, yet little has been done to analyze the parameters required to obtain a robust systemic RNAi response. The data provided here illustrates that in *Tribolium* the concentration and length of dsRNA play a profound effect on the effectiveness of the RNAi response both in regard to initial efficiency and duration of the effect. Additionally, we demonstrate that competitive inhibition of dsRNA can occur when multiple dsRNAs are injected together, influencing the effectiveness of RNAi. These data together provide specific information essential to the design and implementation of RNAi based studies in *Tribolium* and hopefully provokes thought about RNAi studies in other systems.

Introduction

RNA interference (RNAi) is a mechanism of gene silencing triggered by double-stranded RNA (dsRNA) (Fire et al. 1998). dsRNA mediates translational repression through mRNA cleavage or mRNA antisense suppression and transcriptional repression through DNA modification (Lippman and Martienssen 2004; Mello and Conte 2004). While the RNAi pathway is an endogenous pathway known to be involved in regulating eukaryotic gene expression, it has also been harnessed as a genetic tool to inhibit gene expression through mRNA cleavage. This pathway is initiated by the RNaseIII nuclease Dicer, which cleaves dsRNA into 21-23 bp fragments termed short interfering RNAs (siRNA) (Bernstein et al. 2001; Knight and Bass 2001; Carmell and Hannon 2004). The siRNAs are then bound by a complex of proteins known as the RNA induced silencing complex (RISC) (Hammond et al. 2000; Filipowicz 2005). This complex binds mRNA complementary to the siRNA and through the action of the catalytic Argonaute protein causes mRNA cleavage (Tabara et al. 1999; Fagard et al. 2000; Hammond et al. 2001; Parker and Barford 2006). The cleavage of mRNA reduces the amount of mRNA available for translation and thus mimics a loss of function mutation.

The RNAi phenomenon has been described and used as a genetic tool in classical genetic model organisms for over a decade, and recently there has been a barrage of publications illustrating that RNAi is an effective tool in a many emerging model systems as well (Sanchez Alvarado and Newmark 1999; Hughes and Kaufman 2000; Blandin et al. 2002; Bucher et al. 2002; Amdam et al. 2003; Zhu et al. 2003; Tomoyasu and Denell 2004; Boisson et al. 2006; Lynch and Desplan 2006; Zawadzki et al. 2006). However, many studies in these emerging model systems are limited in scope, with most of the data illustrating an RNAi effect for a limited number of genes, in specific tissues, at particular life stages. Because RNAi is a

relatively new tool and has limited uses in *Drosophila* (Miller et al. 2008), there have been few investigations into the parameters required to make RNAi successful in insects. Furthermore, use of RNAi in mammals is difficult due to the interferon response, which can be trigged by dsRNA and can result in cell death (Alexopoulou et al. 2001). Therefore, the vast majority of data available for the proper design of RNAi experiments is from one animal model system, the nematode *Caenorhabditis elegans*. The aim of this work is to provide information helpful for the experimental design of RNAi projects in *Tribolium* and other insect models. Our results show that the size and concentration of dsRNA play an essential role in the effectiveness of the RNAi response, with longer dsRNA being more effective with respect to initial knockdown and duration of the RNAi effect. We also find that when multiple dsRNAs are injected, competition between dsRNAs can occur resulting in a less effective RNAi response. The study of these basic features of RNAi in *Tribolium* will not only aid in experimental design but will also provide insight into the molecular mechanism of the systemic RNAi response in the red flour beetle.

Materials and Methods

Beetle strains

Two transgenic lines of beetles were used in these studies. Pu ll beetles (Lorenzen et al. 2003; Tomoyasu and Denell 2004) are an enhancer trap line in which EGFP is expressed in the nervous system of first instar larvae, in the wing discs of last instar larvae and in the eyes and wings of pupae. AT[11] is a transgenic line in which EGFP is driven by an α tubulin promoter (Siebert et al. 2008). In the AT[11] line EGFP is expressed ubiquitiously at all life stages.

dsRNA synthesis

Template preparation for dsRNA synthesis of *Ubx* and *DsRed* was performed by PCR. The primer was designed against the pCR4-TOPO vector sequence flanking the insertion site, with a T7 promoter sequence at the 5' end as described previously (Tomoyasu et al. 2008). Template preparation for longer *EGFP* dsRNA (520bp and 69bp) synthesis was performed by PCR using template gene specific forward and reverse primers with a T7 promoter sequence at their 5' ends as described previously (Tomoyasu and Denell 2004). For the shorter *EGFP* dsRNA fragments (30bp and 31bp) EGFP template was not used. Instead overlapping primers corresponding to a small region of the *EGFP* coding region were designed with T7 promoter sequence at their 5' ends (Table 1). These overlapping primers were dimerized in a PCR reaction mix at 50C for 20 minutes. This method was chosen to avoid the possibility of synthesizing longer dsRNA than intended due to primers binding to the template in a non-specific manner. dsRNA was synthesized using the MEGAscript T7 High Yield Transcript kit (Ambion). Silencer GFP (eGFP) siRNA (21 bp) was purchased (Ambion).

Injection

Larvae were injected as described previously (Tomoyasu and Denell 2004). For each experimental condition 20-40 larvae were injected from one dsRNA preparation (see supplemental tables for exact numbers and survival rates). dsRNA was injected at a concentration of 1ug/ul (~0.5ug/larva) unless otherwise stated. Coinjection of the 8 fragments of 30bp dsRNA was performed at a total concentration of 1ug/ul (therefore the concentration of each 30bp dsRNA was 0.125ug/ul). Competition experiments sometimes involved two separate injections. The second injection was performed 48 hours after the first injection. Molar dilutions were determined by calculating the molecular weight of dsRNA. For these calculations it was assumed that each of the nucleotide bases were equally represented (therefore 1,000 bp of dsRNA 0.73 ug = 1 pmol). After injection, larvae were maintained on culture flour at 30C.

Documentation

For documentation *Tribolium* larvae were sifted from the flour and submerged in water, which causes the larvae to stop moving, and if they are removed from the water within several hours, is not lethal. After documentation the larvae were removed from the water and dried briefly on a Kimwipe before being returned to the culture flour. Larvae were monitored for EGFP expression 5 days after the initial injection. Pupae were documented 12 days after the initial larval injection. For the duration experiment, adults were documented weekly. Larvae, pupae and adults were documented using an Olympus SZX12 microscope and a Nikon DXM 1200F digital camera. Identical exposure times were used for all the images in one experiment.

Results

dsRNA size

For RNAi experiments, the length of dsRNA used varies widely depending on the model organism (generally ranging from 21 to 1,000 base pairs). In plants both long dsRNAs and siRNAs are effective in eliciting an RNAi response, as both have been shown to spread, silencing both locally and systemically (Klahre et al. 2002). In mammalian cell culture, long dsRNAs induce the interferon response resulting in cell death (Alexopoulou et al. 2001), but small dsRNAs are not recognized by the immune system and thus can be used to achieve gene knockdown (Elbashir et al. 2001). Therefore, either short hairpin RNAs (shRNAs) are engineered and expressed within the cells (Brummelkamp et al. 2002; Paddison et al. 2002) or short interfering RNAs (siRNAs) are transfected into cells (Elbashir et al. 2001). In *Drosophila* S2 cells, long dsRNAs are efficiently taken up by the cell itself (Clemens et al. 2000). However siRNAs are not taken up in S2 cells without the aid of a transfection reagent (Saleh et al. 2006). In *C. elegans*, the animal model in which RNAi has been best studied, it is known that siRNAs are not efficiently transported from cell to cell (Tabara et al. 1999; Parrish et al. 2000; Grishok et al. 2001; Tijsterman et al. 2002). Therefore, in order for an effective RNAi response to be achieved long dsRNAs are used (the minimum length for efficient RNAi in *C. elegans* is between 50 and 100bp) (Parrish et al. 2000). In insects the size range of effective dsRNA has not been fully investigated. Most reports of RNAi use long dsRNA, although there have been limited reports of successful siRNA use in insects (including the pea aphid and the termite) (Mutti 2006; Zhou et al. 2006).

To test the size requirements for dsRNA in *Tribolium* we injected long dsRNAs or siRNAs corresponding to *EGFP* into the Pu 11 transgenic line (Lorenzen et al. 2003; Tomoyasu

and Denell 2004), which expresses EGFP in the nervous system of first larval instars, in the wing discs at the last larval stage and in the eyes and wings at the pupal stage. When long dsRNA (520bp) was injected into last instar larvae, efficient knockdown of EGFP was seen in 100% of individuals at both the larval and pupal stages (n=29) (Fig 1C, STable 1). However, when an siRNA (21bp) (silencer RNA, Ambion) corresponding to *EGFP* was used, normal levels of EGFP expression were observed in every injected individual (n=28) (Fig 1E, STable 1), suggesting that the use of longer dsRNA is necessary to achieve efficient gene knockdown in *Tribolium*.

To further define the size of dsRNA necessary to achieve efficient EGFP knockdown, we synthesized an intermediate size dsRNA (69bp) and a short dsRNA (31bp). Our results indicate that the 69bp fragment was efficient in knocking down EGFP in all individuals (n=8) (Fig 1D, STable 1) while the 31bp fragment was not (n=20) (Fig 1F, STable 1). The results of the siRNA and 31bp dsRNA injections suggest that short dsRNAs are not effective for gene knockdown in *Tribolium*. However, it is known that not all siRNAs are equally efficient (Holen et al. 2002; Kurreck 2006) therefore it may be the specific sequence rather than the size that is ineffective. Alternatively, short dsRNAs may not be recognized by the dsRNA cellular uptake machinery and are therefore not readily incorporated into the cell.

To determine whether the lack of sequence variety was causing the short dsRNAs to be ineffective, we increased the sequence variety by synthesizing a second 31bp dsRNA and coinjecting the two 31bp dsRNA fragments. Together, the two 31bp dsRNAs cover almost the entire region of EGFP targeted by the effective 69bp fragment (Fig 1A). However, these two fragments were also incapable of knocking down EGFP expression, as EGFP expression was still seen in 100% of individuals (n=19) (Fig 1G, STable 1). To further increase the sequence variety,

we synthesized eight overlapping ~30bp dsRNAs spanning 100bp of the EGFP coding region (Fig 1A). In this way, we were able to drastically increase the sequence variety without increasing the size of the dsRNA. When these eight ~30bp dsRNA fragments were injected into *Tribolium* larvae (n=21) they were still unable to knock down EGFP (Fig 1H, STable 1), suggesting that it is not the lack of sequence variety that is causing the ineffectiveness. To determine whether the ineffective RNAi response was due to inefficient uptake by the cells we injected the eight ~30bp fragments into *Tribolium* embryos at the syncytial blastoderm stage. At this stage of embryogenesis cell membranes have not yet formed around the multiple nuclei and therefore the dsRNA is being injected directly into a cell. After the larvae hatched from the eggs they were monitored for EGFP expression in the nervous system. We found that the eight ~30bp fragments were capable of knocking down EGFP expression when injected directly into the egg, as 89% of the hatched larvae showed no EGFP expression (n=16) (Fig 1I, STable 1). These data support the hypothesis that small dsRNAs, are ineffective at multicellular stages in *Tribolium* because they are unable to be taken up by the cells.

dsRNA concentration

We also wanted to determine what concentrations of dsRNA are effective in *Tribolium*. We performed a serial dilution (from 1ug/ul) of *EGFP* dsRNA (520bp) and injected the dsRNA into Pu 11 larvae (Fig 2A). We saw a complete absence of EGFP expression in all injected individuals at concentrations as low as 0.001ug/ul (1,000 fold dilution) (Fig 2A1, A2, STable 2). At a concentration of 0.0001ug/ul (10,000 fold dilution) EGFP expression was reduced (Fig 2A3, STable 2) and at a concentration of 0.00001ug/ul (100,000 fold dilution) EGFP expression appeared comparable to wildtype levels (Fig 2A4, STable 2).

Next we asked whether the size of dsRNA injected influences the effectiveness of EGFP knockdown at low concentrations. We performed a serial dilution (from 1ug/ul) of *EGFP* dsRNA (69bp) (Fig 2B), as this was the smallest size of dsRNA we tested that worked efficiently at a higher concentration. As seen with the 520bp dsRNA the 69bp dsRNA resulted in complete absence of EGFP expression in most individuals at concentration as low as 0.001ug/ul (1,000 fold dilution) (Fig 2B1, B2, STable 2). However, by 0.0001ug/ul the EGFP expression appeared comparable to normal levels in 100% of individuals (10,000 fold dilution) (Fig 2B3, STable 2). This suggests that the 69bp dsRNA may be slightly less effective than the longer 520bp fragment, although finer scale dilutions need to be performed to determine when the 69bp fragment begins to lose effectiveness.

The dilutions described above were all calculated based on dsRNA weight. Longer dsRNA weighs more per molecule than shorter dsRNA. Therefore, when serial dilutions are based on weight the longer dsRNA dilutions will have fewer initial dsRNA molecules than the shorter dsRNA dilutions. However, in the RNAi pathway the dsRNA is cleaved into siRNAs, which are the functional units that bind to target message. One longer dsRNA molecule will give rise to more siRNAs than the shorter molecule of dsRNA. Therefore, when calculations are based on weight, the initial number of dsRNA molecules will differ between the 520bp and 69bp dsRNA, but the final number of siRNAs should be approximately equivalent.

We questioned whether it is the number of dsRNA molecules introduced or the number of siRNAs produced that determines the RNAi efficiency. To address this question we performed a molar dilution series of the 520bp *EGFP* dsRNA (Fig 2C) such that the number of initial molecules in each dilution was equivalent to the 69 bp dsRNA dilution series described above (Fig 2B). With this dilution series the number of 520bp and 69bp dsRNA molecules

injected was the same, but because the 520bp fragment is longer than the 69bp fragment there were more siRNAs produced in each of the 520bp dilutions. We found that when a molar dilution of the 520bp dsRNA was performed there was no EGFP expression in any of the injected individuals at the 100 fold, 1,000 fold, or 10,000 fold dilutions (Fig 2C1-C3, STable 2). EGFP was not effectively reduced when the 520bp dsRNA was diluted 100,000 times (Fig 2C4, STable 2). Since the 10,000 fold dilution of 69bp dsRNA was not (Fig 2B3) and the 10,000 fold dilution of 520bp dsRNA was (Fig 2C3) able to knock down EGFP, these data suggest that it is not the initial number of dsRNA molecules that determines RNAi efficiency but the number of siRNAs produced.

The data above provide information about the concentration of dsRNA needed to achieve efficient knockdown of EGFP in *Tribolium* wing discs. However, RNAi is not always effective in all tissue types. In *Drosophila* it is known that RNAi is less effective in wing imaginal tissue (Kennerdell and Carthew 2000) and in *C. elegans* some nervous tissue is refractory to RNAi due to the expression of the nuclease Eri-1, which degrades the dsRNA (Kennedy et al. 2004). We have previously shown that virtually all tissues in *Tribolium* larvae and pupae are susceptible to RNAi when dsRNA is used at a high concentration (Miller et al. 2008). However, it is possible that not all tissues require the same amount of dsRNA. To determine if any tissues in *Tribolium* require a higher level of dsRNA we performed a serial dilution of *EGFP* dsRNA (520bp) and injected them into transgenic beetles in which EGFP is driven by the α tubulin promoter causing EGFP expression in all tissues (Siebert et al. 2008) (Fig 2D). Our data suggest that all tissues in *Tribolium* larvae are similarly susceptible to RNAi, as EGFP expression was effectively reduced in all tissues at a concentration of 0.001ug/ul in most individuals (1,000 fold dilution) (Fig 2D1, D2, STable2).

Duration of RNAi effect

The duration of the RNAi effect varies in organisms that exhibit a systemic RNAi response. If dsRNA is not continually expressed within the cell, is not maintained by the cell, or is not continually provided to the organism through continued feeding, soaking, or multiple injections then the RNAi effect may wear off as the dsRNA is depleted (Parrish et al. 2000; Price and Gatehouse 2008). However, in plants and *C. elegans* it is known that dsRNA provided to the cell can be amplified via the action of RNA dependent RNA polymerases (RdRPs) (Dalmay et al. 2000; Mourrain et al. 2000; Smardon et al. 2000; Sijen et al. 2001). This amplification mechanism uses mRNA as template to synthesize more dsRNA, thereby increasing the amount of dsRNA available for the RNAi pathway. For organisms that exhibit a prolonged RNAi effect it has been assumed that an amplification mechanism is needed (Price and Gatehouse 2008). However, available genome screens have been unable to identify RdRPs in most metazoans, including insects (Vienne et al. 2003; Tomoyasu et al. 2008). Therefore, if an amplification method exists in *Tribolium* it is assumed to be via a different mechanism.

Regardless of whether an amplification method exists in *Tribolium,* the RNAi effect appears to be long-lived. While this observation has been made anecdotally, duration has not been quantitatively studied. Here we asked the following questions. How long does the RNAi effect last in *Tribolium* adults? Does either the concentration or size of the dsRNA influence the duration of the RNAi effect?

To determine whether concentration influences RNAi duration we injected the 520bp dsRNA for *EGFP* at two different concentrations (0.01ug/ul and 1ug/ul) into Pu 11 larvae and monitored them weekly for the return of EGFP expression (Fig 3). In *Tribolium* adults the wing EGFP expression is difficult to see so we monitored EGFP expression in the adult eye. At the

lower concentration EGFP expression was first detected in some ommatidia of some individuals 98 days after the injection (n=15) (Fig 3A, 3C, STable 3). By day 175 all individuals were showing EGFP expression in some ommatidia (n=14) (Fig 3A, 3C, STable 3). At the high concentration EGFP expression did not return in any individuals for the 175 days they were monitored (n=11) (Fig 3A, 3B, STable3). These data suggest that in *Tribolium* dsRNA concentration does influence the duration of the RNAi effect, with higher concentrations of dsRNA being more effective.

To determine whether the size of dsRNA influences the duration of the RNAi effect we compared individuals in which either the 69bp or the 520bp fragment were injected at a concentration of 0.01ug/ul (which should give rise to approximately the same number of siRNAs) (Fig 3). As mentioned above when using the 520bp fragment of dsRNA at a concentration of 0.01ug/ul the first individual began to exhibit EGFP expression in some ommatidia on day 98 (n=15) (Fig 3A, 3C, STable 3). In contrast, for those individuals injected with the 69bp fragment at a concentration of 0.01ug/ul, EGFP expression was first seen in some ommatidia 28 days after injection (n=8) (Fig 3A, 3D, STable 3). All individuals of this group expressed EGFP by day 77 (n=7) (Fig 3A, 3D, STable 3). These data suggest that in *Tribolium*, size also influences the duration of the RNAi effect, with longer fragments increasing the duration.

dsRNA competition

Occasionally experiments require the knockdown of multiple genes. In these situations combinatorial delivery of dsRNA can be used to remove the function of multiple genes simultaneously (Kuznicki et al. 2000). However, research has shown that when multiple dsRNA or siRNAs are delivered, oversaturation of the RNAi machinery can occur (Parrish et al. 2000;

Yi et al. 2005; Barik 2006; Grimm et al. 2006). This oversaturation causes several problems. First, because the miRNA and RNAi pathways share components, oversaturation of these components during the RNAi response can result in unintentional inhibition of the miRNA pathway resulting in phenotypes related to a loss of miRNA function. Because miRNAs are essential for growth, development, and tissue homeostasis, this inhibition may result in lethality (Grimm et al. 2006). Second, having a mixture of dsRNA can result in competition between the dsRNAs for RNAi machinery components and/or cell entry and transport components resulting in competitive inhibition. This competitive inhibition results in an inability to knock down multiple genes at the same time (McManus et al. 2002; Hutvagner et al. 2004; Bitko et al. 2005; Formstecher et al. 2006; Koller et al. 2006; Castanotto et al. 2007; Stierle et al. 2007). It has been shown that some siRNAs have greater competition potency than others (Formstecher et al. 2006; Koller et al. 2006; Yoo et al. 2008). Therefore, depending on the combination of dsRNAs used different levels of competition may occur.

To determine at what concentration competitive inhibition occurs in *Tribolium* we injected two dsRNAs simultaneously into Pu 11 larvae. One dsRNA (the competitor) was used at a higher concentration and one dsRNA (the reporter) was used at a lower concentration. For the competitor we used one of two dsRNAs, *dsRed* or *Ultrabithorax* (*Ubx*) (Fig 4). We chose to use these dsRNAs as competitors for several reasons. First, because some siRNAs may have greater competition potency than others it is possible that by using two different dsRNA competitors we may see different results. Second, it is possible that having mRNA targets present may affect the competition level. There is no DsRed expression in the Pu 11 beetles, allowing us to test the competition level when the competitor is an exogenous dsRNA with no mRNA target. Ubx is expressed in the beetle hindwing but not the forewing (Tomoyasu et al.

2005). Therefore, in one wing disc the competitor will have a complementary mRNA target and in the other disc it will not. If the presence of target influences the level of competition, we may see differences between the two wing discs. And third, Ubx gives a very distinct wing phenotype (Tomoyasu et al. 2005) enabling us to assess whether the *Ubx* competitor is efficient at down regulating *Ubx*. In both cases (DsRed and Ubx experiments), *EGFP* is used as our reporter gene. By monitoring the EGFP expression in the wing we can determine whether the competitor (*dsRed* or *Ubx*) is preventing the knockdown of EGFP.

When the competitor dsRNA was injected at 10 fold higher concentration than *EGFP* dsRNA we did not see competition at a level that resulted in inefficient knockdown of EGFP, as EGFP expression was effectively reduced in 100% of individuals (n=23 and n=17) (Fig 4B, STable 4). In contrast, when we injected competitor dsRNA at a 100 fold higher concentration than *EGFP* dsRNA, we did see competition that resulted in inefficient knockdown of EGFP in some individuals (n=18 and n=10) (Fig 4C, STable 4). The same result was seen when either *dsRed* or *Ubx* was used as the competitor, and there was no difference between the EGFP expression in the two wing discs in the Ubx experiment. Therefore, our data suggest that the presence of mRNA targets does not affect the competition level.

When competition between dsRNA happens, it may be occurring at several levels. It may occur during cellular uptake and transport of the dsRNA or it may occur during dsRNA processing and mRNA silencing. We reasoned that if the competition is occurring at the level of dsRNA uptake, sequential injection of the competitor dsRNA and the reporter dsRNA may reduce the amount of competition. We injected dsRNA for either *dsRed* or *Ubx*, waited two days, and then injected dsRNA for *EGFP*. When this delay was introduced between injection of the competitor dsRNA and the *EGFP* dsRNA, we no longer saw competition that resulted in

inefficient knockdown of EGFP in any individual (n=11 and n=19) (Fig 4D, STable 2), even at the 100 fold higher concentration. These data suggest that sequential injection can decease the amount of competition that is occurring between dsRNAs, perhaps because competition is occurring when the dsRNA is entering the cell.

The uptake mechanism of dsRNA in insects is currently unknown. Recently, it has been shown that injected dsDNA can be efficiently taken up by cells and transiently expressed in at least one insect (Isoe et al. 2007). We hypothesized that the uptake method of dsRNA and dsDNA may be related. If the uptake of all nucleic acids occurs by the same mechanism and if the competition we are seeing is occurring at the uptake level, then dsDNA may be able to compete with dsRNA. To test this hypothesis we coinjected dsDNA for *dsRed* and dsRNA for *EGFP* into Pu 11 larvae. The dsDNA was at a 100 fold higher concentration than the dsRNA. At this concentration level when two dsRNAs were coinjected there is competition (Fig 4E1, STable 4), resulting in inefficient EGFP knockdown. In contrast, when dsDNA was used we did not see competition in any of the injected individuals (n=29) (Fig 4E2, STable 4). These results suggest that either the transport mechanism of dsRNA is RNA specific or the competition is not occurring at the cellular uptake level.

Discussion

This research provides information that facilitates the planning and execution of RNAi-based studies. With regard to dsRNA size we established that long dsRNA appears to be the most effective with respect to both the initial knockdown and the duration of the RNAi effect. While both a 69bp and a 520bp dsRNA were capable of resulting in gene knockdown, the 520bp fragment was more effective. Several explanations are possible for why the 69bp dsRNA was less efficient. First, while the 69bp dsRNA will give rise to the same number of siRNAs (when compared to an equal weight of 520bp dsRNA) the types of siRNAs produced will be more limited with regard to sequence. Therefore, the possibility exists that the longer dsRNA is not more effective because it is longer but because it produces a greater variety of siRNAs some of which may be more effective at silencing than the limited number of siRNAs produced by the shorter dsRNA fragment. Another possibility is that while the 69bp dsRNA is most assuredly taken up by the cell, the efficiency of this uptake may not be as high, resulting in a lower quantity of siRNAs available for silencing. Finally, if a dsRNA amplification or storage mechanism occurs in *Tribolium*, these processes may also be affected by dsRNA size.

While dsRNA 69bp and longer did result in gene knockdown, smaller dsRNAs (31 and 21bp) were ineffective at gene silencing when injected into *Tribolium* at a multicellular stage. Our data suggest this is due to inefficient uptake of the shorter dsRNA fragments. Data from *C. elegans* and *Drosophila* S2 cells support this hypothesis (Tabara et al. 1999; Parrish et al. 2000; Grishok et al. 2001; Tijsterman et al. 2002). However, the use of siRNAs has effectively achieved gene knockdown in both the pea aphid and the termite. In these experiments siRNAs were derived from long dsRNA cleaved *in vitro* by the Dicer enzyme (Mutti 2006; Zhou et al. 2006). Therefore, there is a possibility that the siRNA samples contained a mixture of long

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dsRNA and siRNAs. Another, scenario is that insect cells from different species may recognize and/or uptake dsRNA in different ways.

In *C. elegans*, amplification by RNA dependent RNA polymerases (RdRP) is essential to achieve any RNAi effect (Smardon et al. 2000; Sijen et al. 2001). Therefore, it has been assumed that an amplification mechanism is needed in all organisms that exhibit a prolonged RNAi effect. However, not only have RdRPs not been found in insects (Tomoyasu et al. 2008), but it has also been shown that isoform specific RNAi can be performed in *Tribolium* (Arakane et al. 2005), suggesting that amplification using the endogenous mRNA as template is not occurring. There remains a possibility that amplification is occurring through another mechanism (perhaps using the dsRNA as template, as this would still allow for isoform specific RNAi). However, amplification may not be needed to achieve an effective long lasting RNAi effect. It is possible that insects with a robust systemic RNAi effect simply have the ability to efficiently take up and/or store dsRNA.

While the mechanism of RNAi duration in *Tribolium* has not been determined, we were able to show that when dsRNA is injected at the last larval stage the effect can last for many months, perhaps even for the entire lifespan of the individual. It has also been shown that parental RNAi (in which female pupae or adults are injected with dsRNA and the effect is seen in the offspring) can also be effective for several months (Bucher et al. 2002), suggesting an extremely efficient RNAi response. However, we have not seen a parental RNAi effect when last instar larvae are injected with dsRNA (data not shown). In other words, when larvae are injected the next generation is not affected. One explanation for this is that the female reproductive organs do not form until the pupal stage. Perhaps in order for the oocytes to uptake dsRNA they must be formed at the time of dsRNA introduction to the body cavity. If this is true

we might expect to see the RNAi effect lasting longer in established tissue and being less effective in tissue that is continually turned over.

In *C. elegans* an RNAi effect can be seen for multiple generations if the gene being targeted is expressed in the germ cells (Grishok et al. 2000). This is presumably due to repeated amplification of the dsRNA in the germ cells every generation. However, even in *C. elegans*, where there is amplification and dsRNA spreading, if the gene being targeted is not expressed in the germ cells, the RNAi effect does not last more than one generation. This suggests that even in *C. elegans* dsRNA amplification and subsequent spreading is not sufficient to affect new tissues.

In addition to data concerning the effect of dsRNA size and concentration on the duration of the RNAi, the duration experiments revealed several other interesting observations. When the RNAi effect wears off in the adult, EGFP expression appears to return one ommatidia at a time in a mosaic pattern across the surface of the eye. In a process that takes weeks (or even months), more and more ommatidia begin to express EGFP until EGFP in the eye reaches wildtype levels. The pattern of EGFP return suggests that, at least in the eye, the RNAi is acting cell autonomously. Additionally we also observed that there were vast differences in the length of time it took for EGFP to return in one experimental group. For example when the 69bp fragment was used at a concentration of 0.01ug/ul the first individual began to express EGFP 28 days post injection. However, EGFP was not seen in all individuals until 77 days post injection. These differences probably represent subtle differences in injection volume between individuals, but may also reflect variation in the injection site (distance from the eye) or in individual's ability to uptake, store, or amplify the dsRNA.

It is important to note that with regard to the dsRNA concentration and duration experiments the results are probably gene specific. For example, EGFP expression was removed with a concentration of only 0.001ug/ul, which may not be efficient to deplete the expression of other genes. In our lab, RNAi experiments, that are intended to result in complete gene knockdown, are generally performed with dsRNA concentrations varying between 1 and 4ug/ul. While the exact concentration required and the exact number of days an effect will last will probably vary between genes, we expect the trends to remain the same. Longer dsRNA and higher concentrations of dsRNA should result in more efficient gene knockdown and a longer knockdown duration.

Because the uptake, transport and processing of dsRNA all require cellular components and proteins that are finite, competition for these components will occur at some level. The question is, which level is most sensitive to oversaturation. Once dsRNA is injected into the individual, dsRNA that is not taken up into cells is presumably excreted. Therefore, the uptake of dsRNA must occur relatively quickly. However, the duration of the RNAi effect suggests that mRNA silencing occurs for an extended period of time. Therefore, the fact that sequential injection of multiple dsRNA appears to lessen the level of competition suggests that the competition seen in our assay is occurring at the level of dsRNA uptake (although competition at the mechanism level may also occur at particular ratios). Regardless of the step at which competition is occurring, our data does indicate that at certain ratios combinatorial delivery of dsRNA can result in competitive inhibition. As competition potency may vary between dsRNAs this ratio may vary depending on the combination of dsRNAs used.

Our competition experiments did not show any observable impact on the miRNA pathway. miRNAs are essential for growth and development, and specifically are known to be

involved in metamorphosis in insects (Bashirullah et al. 2003). Additionally, we have shown that knockdown of Tc-Argonaute-1 (an essential component of the miRNA machinery) results in larval lethality (Tomoyasu et al. 2008). However, we never saw any lethality or developmental phenotypes indicating that the miRNA pathway was impaired due to oversaturation of miRNA pathway components. In order to inhibit the miRNA pathway higher concentrations of dsRNA may need to be used. It may also be possible that miRNA inhibition by oversaturation of the RNAi machinery may be more difficult to achieve in *Tribolium* due to subfunctionalization of the machinery components. Both *Drosophila* and *Tribolium* appear to have proteins, such as Argonaute-1 and Argonatue-2, that have duplicated and subfunctionalized such that one protein is involved in the miRNA pathway while the other is involved in the RNAi pathway (Forstemann et al. 2007). Therefore, it is possible that in these insects accidental inhibition of the miRNA pathway may occur less often.

Within the last decade RNAi has become a genetic tool that has made the functional study of genes in non-model systems readily available. While there has been a race to identify the next organism in which RNAi can be used, the details have often been overlooked. The work described here provides specific data on the essential parameters for RNAi in the red flour beetle and hopefully brings to light important considerations when planning RNAi experiments in other organisms.

Acknowledgements

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Table 1: Primers used to synthesize *EGFP* dsRNA

Primer	Sequence	dsRNA size
GFPiF2	<u>TAATACGACTCACTATA</u>**G**GGCGATGCCACCT	520bp
GFPiR5	<u>TAATACGACTCACTATA</u>**G**GGCGGACTGGGTG	
GFPiF2	<u>TAATACGACTCACTATA</u>**G**GGCGATGCCACCT	69bp
GFPiR2	<u>TAATACGACTCACTATA</u>**G**GGCACGGGCAGCT	
GFPiF1d	<u>TAATACGACTCACTATA</u>**G**GGCGATGCCACCTACGGCAAG	31bp
GFPIR1d	<u>TAATACGACTCACTATA</u>**G**GGTCAGCTTGCCGTAGGTGGC	
GFPiF1d2	<u>TAATACGACTCACTATA</u>**G**GGATCTGCACCACCGGCAAGCTGCC	31bp
GFPiR1d2	<u>TAATACGACTCACTATA</u>**G**GGAGGGCACGGGCAGCTTGCCGGTG	
FragF2	<u>TAATACGACTCACTATA</u>**G**GGCACCTACGGCAAGCTGACCCTGA	30bp
FragR2	<u>TAATACGACTCACTATA</u>**G**GGATGAACTTCAGGGTCAGCTTGC	
FragF3	<u>TAATACGACTCACTATA</u>**G**GGAAGCTGACCCTGAAGTTCATCTG	30bp
FragR3	<u>TAATACGACTCACTATA</u>**G**GGGTGGTGCAGATGAACTTCAGG	
FragF4	<u>TAATACGACTCACTATA</u>**G**GGTGAAGTTCATCTGCACCACCGGC	30bp
FragR4	<u>TAATACGACTCACTATA</u>**G**GGCAGCTTGCCGGTGGTGCAGAT	
FragF5	<u>TAATACGACTCACTATA</u>**G**GGCTGCACCACCGGCAAGCTGCCCG	30bp
FragR5	<u>TAATACGACTCACTATA</u>**G**GGCAGGGCACGGGCAGCTTGCCGG	
FragF6	<u>TAATACGACTCACTATA</u>**G**GGGCAAGCTGCCCGTGCCCTGGCC	30bp
FragR6	<u>TAATACGACTCACTATA</u>**G**GGAGGGTGGGCCAGGGCACGGGC	
FragF7	<u>TAATACGACTCACTATA</u>**G**GGCCGTGCCCTGGCCCACCCTCGTG	30bp
FragR7	<u>TAATACGACTCACTATA</u>**G**GGGGTGGTCACGAGGGTGGGCCA	
FragF8	<u>TAATACGACTCACTATA</u>**G**GGCCCACCCTCGTGACCACCCTGA	30bp
FragR8	<u>TAATACGACTCACTATA</u>**G**GGCCGTAGGTCAGGGTGGTCACGA	

Underlined represents the minimum promoter sequence for T7 polymerase.
Bold **G** is the first based incorporated into RNA during transcription.

Supplemental Table 1: dsRNA size requirements (Fig 1)

Treatment	#Injected	#Surviving	#GFP+	%GFP+
520bp-larvae	32	29	0	0
69bp-larvae	25	8	0	0
siRNA-larvae	40	28	28	100
31bp-larvae	27	20	20	100
2-31bp-larvae	24	19	19	100
8-~30bp-larvae	26	21	21	100
8-~30bp-eggs	~300	16	2	11
520bp-eggs	~225	13	0	0

Treatment	Strain	#Injected	#Surviving	#GFP+	%GFP+
520bp-0.01ug/ul	Pu11	30	26	0	0
520bp-0.001ug/ul	Pu11	29	23	0	0
520bp-0.0001ug/ul	Pu11	29	25	19	76
520bp-0.00001ug/ul	Pu11	24	19	19	100
69bp-0.01ug/ul	Pu11	26	15	2	13
69bp-0.001ug/ul	Pu11	27	18	2	11
69bp-0.0001ug/ul	Pu11	27	17	17	100
69bp-0.00001ug/ul	Pu11	27	16	16	100
520bp-100molar dilution	Pu11	34	31	0	0
520bp-1000molar dilution	Pu11	23	18	0	0
520bp-10000molar dilution	Pu11	29	20	0	0
520bp-100000molar dilution	Pu11	26	21	21	100
520bp-0.01ug/ul	α tubulin	43	20	0	0
520bp-0.001ug/ul	α tubulin	41	15	4	27
520bp-0.0001ug/ul	α tubulin	35	16	16	100
520bp-0.00001ug/ul	α tubulin	30	11	11	100

Supplemental Table 3: Duration assays (Fig 3)

Treatment	# Injected	GFP+/total Day 14	GFP+/total Day 28	GFP+/total Day 56	GFP+/total Day 77	GFP+/total Day 98	GFP+/total Day 140	GFP+/total Day 175
520bp1ug/ul	26	0/13	0/11	0/11	0/11	0/11	0/11	0/11
520bp0.01ug/ul	24	0/15	0/15	0/15	0/15	4/15	8/14	14/14
69bp0.01ugu/	24	0/10	3/8	5/8	7/7	5/5	5/5	5/5

Treatment	#Injected		#Surviving	#GFP+	%GFP+
dsRed/EGFP 10:1	23		23	0	0
Ubx/EGFP 10:1	17		17	0	0
dsRed/EGFP 100:1	34		18	8	44
Ubx/EGFP 100:1	15		10	6	60
dsRed DNA/EGFP 100:1	34		29	0	0
Treatment	Injection 1	Injection 2 (EGFP)	#Surviving	#GFP+	%GFP+
Sequential dsRed/EGFP 100:1	42	11	11	0	0
Sequential Ubx/EGFP 100:1	51	19	19	0	0

Figure 1

The effect of dsRNA size on RNAi knockdown efficiency. **(A)** Depiction of *EGFP* dsRNAs relative to the *EGFP* coding region. Green: *EGFP* coding region. Red: long dsRNA (520bp). Purple: intermediate dsRNA (69bp). Blue: short dsRNA (30/31bp). **(B)** Uninjected Pu 11 *Tribolium* larvae. **(C-H)** Pu 11 *Tribolium* larvae injected with *EGFP* dsRNA. **(C)** 520bp dsRNA. n=29 **(D)** 69bp dsRNA. n=8 **(E)** 21bp siRNA. n=28 **(F)** 31bp dsRNA. n=20 **(G)** Two 31bp dsRNAs. n=19 **(H)** Eight ~30bp dsRNAs. n=21 **(I,J)** Pu 11 *Tribolium* larvae injected as embryos with *EGFP* dsRNA. **(I)** Top larvae; uninjected, Middle larvae; injected with eight ~30bp dsRNAs (n=16), Bottom larvae; injected with 520bp dsRNA (n=13). **(J)** Light microscopy image of **(I)**.
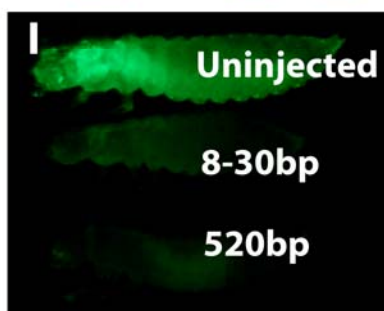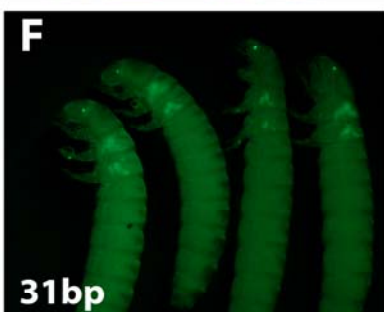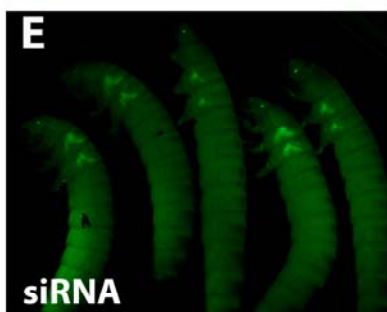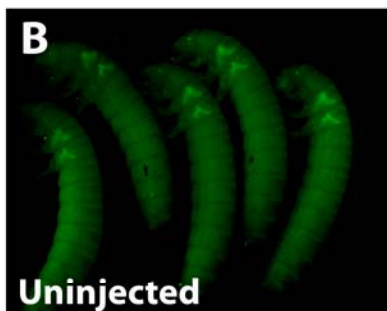
A EGFP (1-720bp)

520bp (104-623bp)

69bp (104-172bp)

31bp (142-172bp)

31bp (104-134bp)

30bp (114-143bp)

30bp (124-153bp)

30bp (134-163bp)

30bp (144-173bp)

30bp (154-183bp)

30bp (164-193bp)

30bp (174-203bp)

B Uninjected

C 520bp

D 69bp

E siRNA

F 31bp

G 2-31bp

H 8-30bp

I Uninjected / 8-30bp / 520bp

J Uninjected / 8-30bp / 520bp
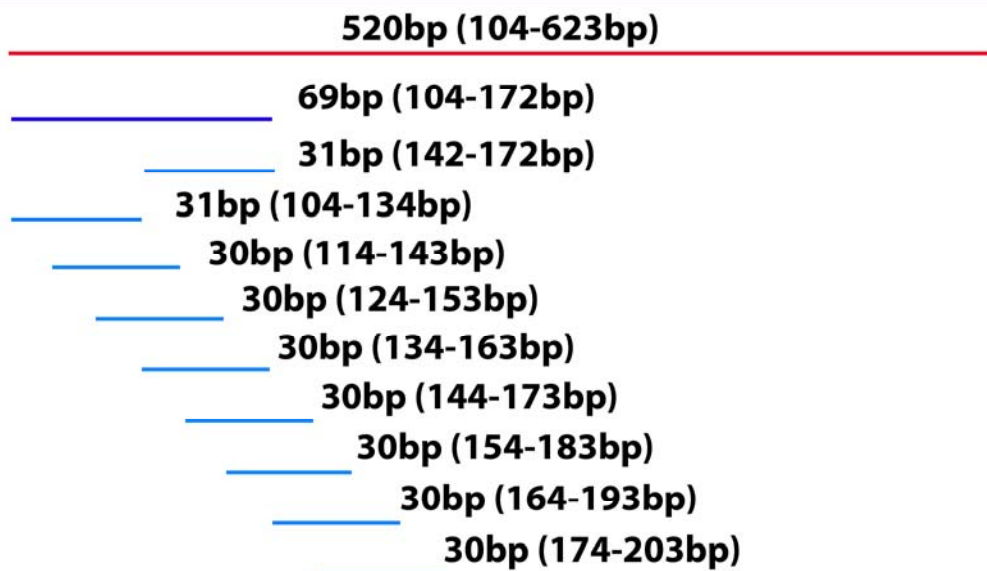
Figure 2

The effect of dsRNA concentration on RNAi knockdown efficiency. **(A)** Weight dilution series of 520bp *EGFP* dsRNA injected into Pu 11 *Tribolium* larvae. **(1)** 0.01ug/ul. n=26 **(2)** 0.001ug/ul. n=23 **(3)** 0.0001ug/ul. n=25 **(4)** 0.00001ug/ul. n=19 **(B)** Weight dilution series of 69bp *EGFP* dsRNA injected into Pu 11 *Tribolium* larvae. **(1)** 0.01ug/ul. n=15 **(2)** 0.001ug/ul. n=18 **(3)** 0.0001ug/ul. n=17 **(4)** 0.00001ug/ul. n=16 **(C)** Molar dilution series of 520bp *EGFP* dsRNA injected into Pu 11 *Tribolium* larvae. **(1)** ~0.07ug/ul. n=31 **(2)** ~0.007ug/ul. n=18 **(3)** ~0.0007ug/ul. n=20 **(4)** ~0.00007ug/ul. n=21 **(D)** Weight dilution series of 520bp *EGFP* dsRNA injected into α tubulin EGFP *Tribolium* larvae. **(1)** 0.01ug/ul. n=20 **(2)** 0.001ug/ul. n=15 **(3)** 0.0001ug/ul. n=16 **(4)** 0.00001ug/ul. n=11
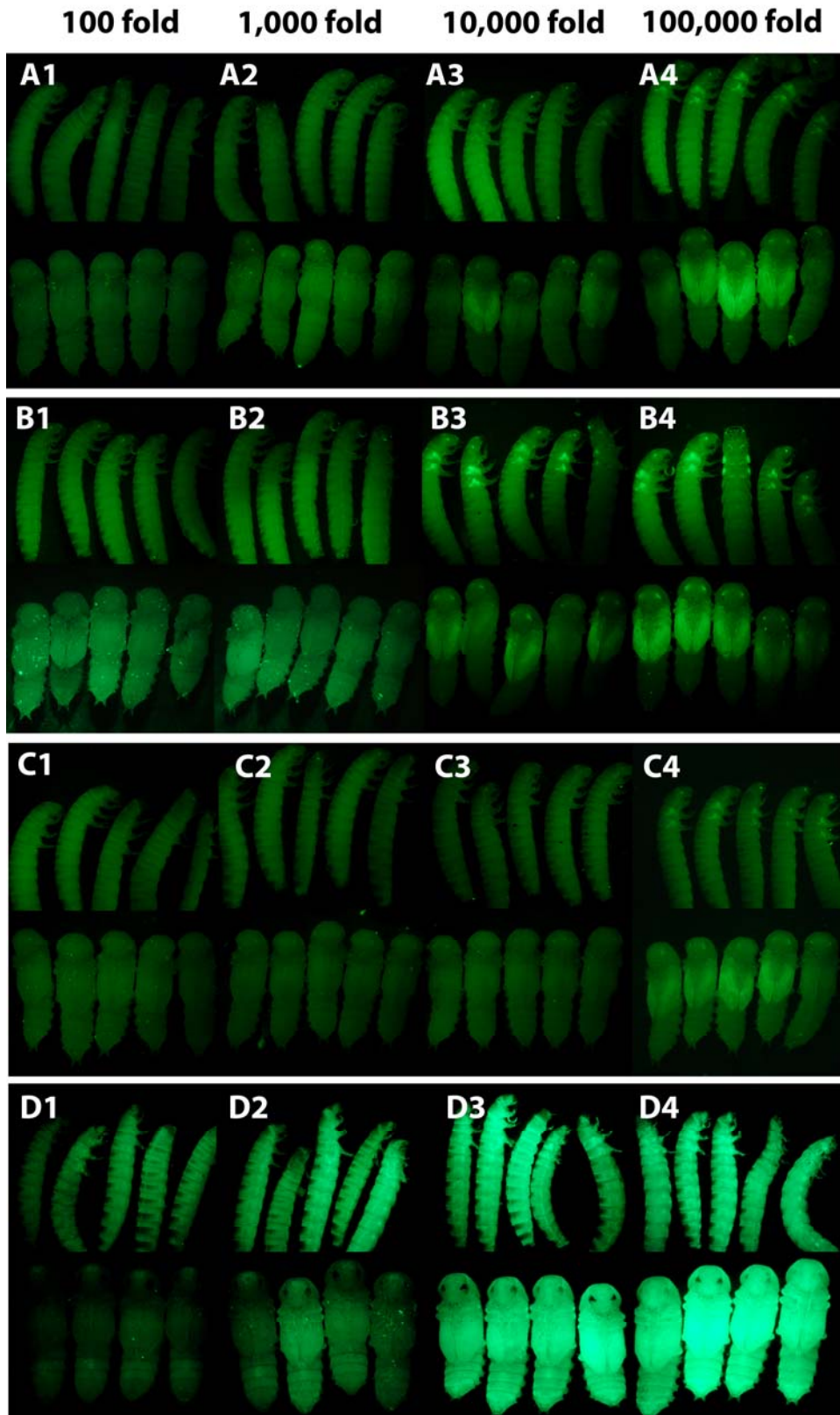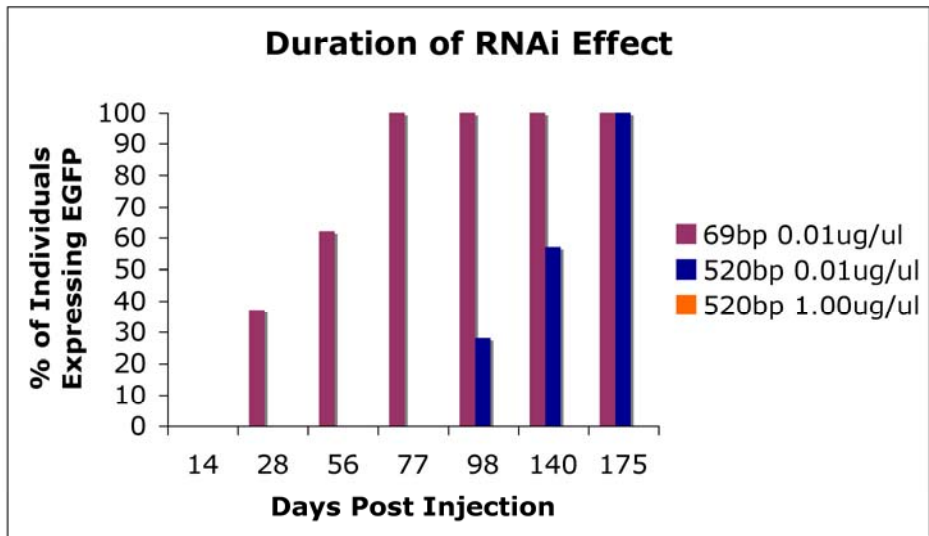
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Figure 3

The effect of dsRNA size and concentration on the duration of the RNAi effect. **(A)** Bar graph depicting the percentage of individuals expressing EGFP after injection of *EGFP* dsRNA at two different concentration and sizes. **(B)** Pu 11 *Tribolium* injected with 520bp *EGFP* dsRNA at a concentration of 1ug/ul. n=11 **(C)** Pu 11 *Tribolium* injected with 520bp *EGFP* dsRNA at a concentration of 0.01ug/ul. n=15 **(D)** Pu 11 *Tribolium* injected with 69bp *EGFP* dsRNA at a concentration of 0.01ug/ul. n=8. Asterik indicates the first day EGFP expression was detected in the adult eye.

A

Duration of RNAi Effect

Legend:
- 69bp 0.01ug/ul
- 520bp 0.01ug/ul
- 520bp 1.00ug/ul

Y-axis: % of Individuals Expressing EGFP
X-axis: Days Post Injection (14, 28, 56, 77, 98, 140, 175)

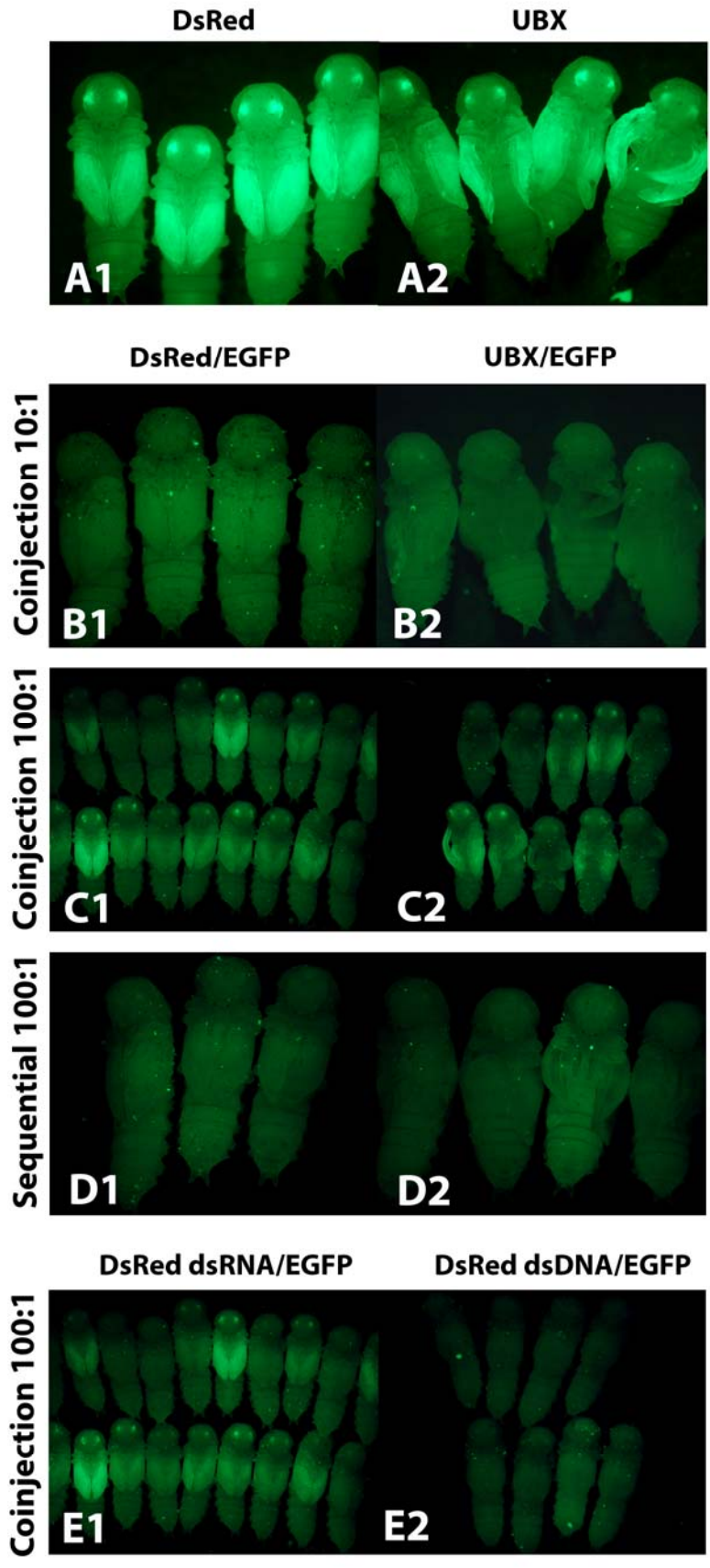| Day 14 | Day 28 | Day 56 | Day 77 | Day 98 | Day 140 | Day 175 |

B

520bp 1ug/ul

C

520bp 0.01ug/ul

D

69bp 0.01ug/ul

Figure 4

The effect of dsRNA competition on RNAi knockdown efficiency. **(A)** Competitor dsRNA injected alone. **(1)** *DsRed* dsRNA. **(2)** *Ubx* dsRNA. **(B)** Competitor and reporter dsRNA coinjected at a 10 to 1 ratio. **(1)** *DsRed* dsRNA injected at 1ug/ul (competitor). *EGFP* dsRNA injected at 0.1ug/ul (reporter). n=23 **(2)** *Ubx* dsRNA injected at 1ug/ul (competitor). *EGFP* dsRNA injected at 0.1ug/ul (reporter). n=17 **(C)** Competitor and reporter dsRNA coinjected at a 100 to 1 ratio. **(1)** *DsRed* dsRNA injected at 1ug/ul (competitor). *EGFP* dsRNA injected at 0.01ug/ul (reporter). n=18 **(2)** *Ubx* dsRNA injected at 1ug/ul (competitor). *EGFP* dsRNA injected at 0.01ug/ul (reporter). n=10 **(D)** Competitor and reporter dsRNA injected sequentially at a 100 to 1 ratio. **(1)** *DsRed* dsRNA injected at 1ug/ul (competitor). *EGFP* dsRNA injected at 0.01ug/ul (reporter). n=11 **(2)** *Ubx* dsRNA injected at 1ug/ul (competitor). *EGFP* dsRNA injected at 0.01ug/ul (reporter). n=19 **(E)** *DsRed* dsRNA or *DsRed* dsDNA is used as the competitor nucleic acid. *EGFP* dsRNA is coinjected as the reporter. The ratio of competitor to reporter is 100 to 1. **(1)** *DsRed* dsRNA injected at 1ug/ul. *EGFP* dsRNA injected at 0.01ug/ul. n=18 **(2)** *DsRed* dsDNA injected at 1ug/ul. *EGFP* dsRNA injected at 0.01ug/ul. n=29

DsRed UBX

A1 A2

DsRed/EGFP UBX/EGFP

Coinjection 10:1

B1 B2

Coinjection 100:1

C1 C2

Sequential 100:1

D1 D2

DsRed dsRNA/EGFP DsRed dsDNA/EGFP

Coinjection 100:1

E1 E2

Chapter II

Larval RNAi in *Drosophila*?

Running head: Larval RNAi in *Drosophila*

Sherry C. Miller, Susan J. Brown and Yoshinori Tomoyasu*
Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506

Keywords: RNAi, systemic, *Tribolium castaneum* (red flour beetle), *Drosophila melanogaster* (fruit fly), injection

*Corresponding author: Yoshinori Tomoyasu**, Division of Biology Kansas State University, Ackert Hall, Manhattan, KS 66502
**Current contact information: Department of Zoology, Miami University, 212 Pearson Hall, Oxford, OH 45056, Phone (513) 529-3162, email: tomoyay@muohio.edu

Abstract

RNA interference (RNAi) has become a common method of gene knockdown in many model systems. To trigger an RNAi response, dsRNA must enter the cell. In some organisms, such as *Caenorhabditis elegans*, cells can take up dsRNA from the extracellular environment via a cellular uptake mechanism termed systemic RNAi. However, in the fruit fly *Drosophila melanogaster*, it is widely believed that cells are unable to take up dsRNA, although there is little published data to support this claim. In this study we set out to determine whether this perception has a factual basis. We took advantage of traditional Gal4/UAS transgenic flies as well as the MARCM system to show that extracellular injection of dsRNA into *Drosophila* larvae cannot trigger RNAi in most *Drosophila* tissues (with the exception of hemocytes). Our results show that this is not due to a lack of RNAi machinery in these tissues as overexpression of dsRNA inside the cells using hairpin RNAs efficiently induces an RNAi response in the same tissues. These results suggest that while most *Drosophila* tissues indeed lack the ability to uptake dsRNA from the surrounding environment, hemocytes can initiate RNAi in response to extracellular dsRNA. We also examined another insect, the red flour beetle *Tribolium castaneum*, which has been shown to exhibit a robust systemic RNAi response. We show that virtually all *Tribolium* tissues can respond to extracellular dsRNA, which is strikingly different from the situation in *Drosophila*. Our data provide specific information about the tissues amenable to RNAi in two different insects, which may help us understand the molecular basis of systemic RNAi.

Introduction

Since its initial characterization in *Caenorhabditis elegans*, RNA interference (RNAi) has become a powerful genetic tool in many organisms, allowing the knockdown of homologous gene products by the introduction of dsRNA into cells (May and Plasterk 2005). Introduction can be achieved by a variety of methods including microinjection, electroporation, and hairpin RNA expression (May and Plasterk 2005). However, for some organisms, such as *C. elegans*, artificial introduction of dsRNA directly into cells is not required (Fire et al. 1998). In these organisms the dsRNA is taken up from the extracellular environment (and spread from cell to cell) via a cellular uptake mechanism termed systemic RNAi (Fire et al. 1998).

In recent years, a variety of insects, including the red flour beetle *Tribolium castaneum* (Bucher et al. 2002; Tomoyasu and Denell 2004), have been shown to exhibit systemic uptake of dsRNA (sometimes in limited tissues) (see Tomoyasu et al. 2008 for more details about insect species that show a systemic RNAi response). However, for the well-established insect model system, *Drosophila melanogaster*, there is a widely held belief that its cells are unable to take up dsRNA in a systemic manner. While this perception remains entrenched in the fly community, little empirical data has been published to support this claim. In fact, there are published reports of successful RNAi by injection of dsRNA in *Drosophila* adults (Dzitoyeva et al. 2001; Goto et al. 2003; Petruk et al. 2006).

In this study we set out to determine whether the belief that *Drosophila* cells are unable to take up dsRNA from their environment holds true. For comparison, we also examined dsRNA uptake in *Tribolium castaneum*. We find that systemic RNAi in larval tissues indeed differs drastically between these two insect species. While virtually all larval tissues in *Tribolium* are

able to take up dsRNA and mount an RNAi response, only one larval cell type in *Drosophila*, the hemocyte, is able to respond to injected extracellular dsRNAs.

Materials and methods

Fly stocks and crosses

Flies were raised at 25°C. The Gal4 lines used in this study and their expression patterns are summarized in Table 1. These lines were crossed to either *UAS-EGFP/TM3* or *UAS-GFP*^{S65T} to obtain GFP expressing larvae for injection. The *UAS-EGFP-RNAi* line, (*w; P(UAS-Avic/GFP.dsRNA.R)143* (Roignant et al. 2003), was also used to induce the RNAi response in these EGFP expressing larvae. *Tub-Gal4; Tub-Gal80* flies (*w; tubP-Gal80; tubP-Gal4/TM6B, Tb*) (Lee and Luo 2001) were crossed with *UAS-GFP*^{S65T} for the Gal80 RNAi experiment.

Beetle strains

Beetles were cultured at 30°C on whole wheat (+5% yeast) flour. The strain AT^{11}, in which EGFP expression is driven ubiquitously by the *aTub* promoter (Siebert et al. 2008), was used for injection.

dsRNA synthesis

Double-stranded RNA was synthesized using the Ambion MEGAscript high yield transcription kit. Template for the synthesis of EGFP dsRNA was prepared by PCR using gene specific primers with a T7 polymerase promoter sequence at the 5' ends as described by (Tomoyasu and Denell 2004). Templates for the synthesis of *GFP* and *Gal80* dsRNA were prepared by PCR using vector specific primers with a T7 polymerase promoter sequence at the 5' ends as described by (Tomoyasu et al. 2008).

Injection into beetle larvae

Injection into beetle larvae was performed as previously described by (Tomoyasu and Denell 2004). dsRNA was injected at a concentration of 1ug/ul (approximately 0.5ug per larva).

Injection into fly larvae

Last instar larvae expressing the reporter gene were selected. Larvae were dried on filter paper and then immobilized on double stick tape adhered to a microscope slide. The larvae were positioned ventral side down and were injected on their dorsal side in an anterior to posterior direction. dsRNA for *EGFP* and *GFP* was injected at a concentration of 1ug/ul (less than 0.25ug per larva). dsRNA for *Gal80* was injected at a concentration of 2ug/ul (less than 0.5ug per larva). Larvae were removed from the slide after injection and raised at 25°C until analysis.

Results and discussion

To determine whether *Drosophila* larval cells have the ability to take up dsRNA and execute an RNAi response, we employed the Gal4/UAS system (Brand and Perrimon 1993) to express a reporter gene (*GFP* or *EGFP*) in a variety of *Drosophila* tissues (Table 1, Fig 1 A-F left, Fig 2 A-B left and middle). We then injected third instar larvae with dsRNA for the reporter gene and monitored reporter gene expression 24 and 48 hours (data not shown) after injection. Reporter gene expression was maintained in most of the tissues tested including wing discs, fat bodies, salivary glands, muscles, midgut, brain and ectoderm (Fig 1 A-F middle). Intriguingly, reporter gene expression was lost in only one of the cell types tested, the hemocytes (Fig 2 A-B right). A mock injection (dsRNA for *dsRed*) did not induce the reduction of reporter gene expression in hemocytes (data not shown), indicating that the dsRNA silencing in hemocytes is not a non-specific effect induced by dsRNA molecules. Our findings indicate that introducing dsRNA by injection in the last larval stage is ineffective at triggering RNAi in many tissues. However, *Drosophila* hemocytes (visualized by *pxn-Gal4/UAS GFP* (Stramer et al. 2005)) do appear to have the ability to take up dsRNA and perform RNAi *in vivo*.

To confirm our results and test other *Drosophila* tissues for a systemic RNAi response, we took advantage of the MARCM system (Lee and Luo 2001). This system uses Gal80 as a repressor of Gal4 to prevent expression normally produced by the Gal4/UAS system (Lee and Luo 2001). We created a line in which GFP expression was driven by UAS, and both Gal80 and Gal4 were expressed ubiquitously by the *tubulin* promoter (Table 1). In these flies there is no GFP expression due to the repression of Gal4 by Gal80 (Fig 3B middle). We attempted to relieve Gal4 repression by injecting dsRNA for *Gal80* into last instar larvae. When GFP expression was monitored 24 and 72 hours after injection, hemocytes were the only tissue in

which Gal4 repression appeared to be relieved (Fig 3B right). These data confirm our initial results, suggesting that hemocytes are the only apparent tissue capable of taking up dsRNA and mounting an RNAi response.

To determine whether the ineffective RNAi response in most *Drosophila* tissues was due to a lack of intracellular RNAi machinery or due to a more upstream process, we expressed *EGFP* hairpin RNAs to trigger RNAi inside the cells (Roignant et al. 2003) (Table 1). We observed a reduction of EGFP expression in all tissues examined (Fig 1 A-F right), indicating that the lack of RNAi response after injection of dsRNA was not due to defects in the RNAi machinery. These results lend support to the conclusion that failure in upstream events in the systemic RNAi pathway, such as dsRNA cellular uptake, transport, or maintenance, is responsible for the ineffective RNAi in most *Drosophila* larval tissues.

Recent reports illustrate that post-embryonic injection of dsRNA in other non-drosophilid insects can result in a systemic RNAi effect. However, in many of these organisms, only specific tissues, such as epidermal tissues or fat bodies, have been examined (summarized in (Tomoyasu et al. 2008)). To reveal whether all tissues in *Tribolium* have the capacity to take up dsRNA, we utilized a transgenic *Tribolium* line in which *EGFP* is driven ubiquitously by the native *alpha tubulin (aTub)* promoter (Siebert et al. 2008). Last instar larvae were injected with dsRNA for *EGFP* and then monitored for EGFP expression 48 and 96 hours later. In contrast to the *Drosophila* results, EGFP expression was reduced or absent in virtually all *Tribolium* tissues at both larval and pupal stages (Fig 4A-F). In most *Tribolium* tissues, EGFP expression appears completely absent. However, some residual EGFP expression is still seen in the ventral portion of each larval segment (ganglia) (arrow in Fig 4D) and in the pupal brain (arrow in Fig 4F). The remaining EGFP expression may be due to differences in initial expression rather than tissues

resistance to RNAi as both of these tissues expressed EGFP at a higher level than surrounding tissues prior to injection. The higher level of initial EGFP expression in the brain and ganglion may require more time and/or dsRNA to achieve efficient knockdown. Alternatively, there still is a possibility that some *Tribolium* neural tissues may be somewhat resistant to RNAi. These results indicate that virtually all *Tribolium* larval tissues have the ability to take up dsRNA from the extracellular environment and mount an RNAi response.

Our data provide insight into the use of RNAi as a tool for the study of post-embryonic development in insects. While larval injection of dsRNA is not effective for many *Drosophila* tissues, some successful reports of adult injection (Dzitoyeva et al. 2001; Goto et al. 2003; Petruk et al. 2006) may suggest different tissue specificity at different developmental stages. The basis of this difference between larval and adult tissues is unknown at this time but may be due to fundamental developmental differences between tissue types, such as cell ploidy, or due to differences in gene expression required for the uptake and transport of dsRNA. While most *Drosophila* larval tissue in not susceptible to dsRNA by injection our data does reveal a potentially powerful application for RNAi in the study of hemocyte development and their role in insect immunity. In addition, *Tribolium's* ability to efficiently perform RNAi in virtually all cell types makes it an attractive insect model for the study of post-embryonic development and the systemic RNAi response itself.

Acknowledgements

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Table 1: Transgenic *Drosophila* lines

Gal4 Line	Expression	Genotypes for Injection	dsRNA	Hairpin Genotype
w; P{GawB}48Y	brain salivary glands	*w; P{GawB}48Y/+;* *P{UAS-EGFP}34/+*	eGFP	*w; P{GawB}48Y/ P{UAS-Avic/GFP.* *dsRNA.R}143; P{UAS-EGFP}34/+*
y¹w; P{en2.4-Gal4}e16E *P{UAS-FLP1.D}JD1*	segmental ectoderm	*w; P{en2.4-Gal4}e16EP{UAS-FLP1.* *D}JD1/+; P{UAS-EGFP}34/+*	eGFP	*w; P{en2.4-Gal4}e16E P{UAS-FLP1.D}JD1/ P{UAS-* *Avic/GFP.dsRNA.R}143; P{UAS-EGFP}34/+*
w; P{GawB}c179	muscles salivary glands	*w; P{GawB}c179/+;* *P{UAS-EGFP}34/+*	eGFP	*w; P{GawB}c179/ P{UAS-Avic/GFP.* *dsRNA.R}143; P{UAS-EGFP}34/+*
w¹¹¹⁸; P{Cg-GAL4.A}2	fat bodies	*w; P{Cg-GAL4.A}2/+;* *P{UAS-EGFP}34/+*	eGFP	*w; P{Cg-GAL4.A}2/ P{UAS-Avic/GFP.* *dsRNA.R}143; P{UAS-EGFP}34/+*
y¹w¹¹¹⁸; P{ey1x-GAL4.Exel}2	midgut salivary glands	*w; P{ey1x-GAL4.Exel}2/+;* *P{UAS-EGFP}34/+*	eGFP	*w; P{ey1x-GAL4.Exel}2/ P{UAS-Avic/* *GFP.dsRNA.R}143; P{UAS-EGFP}34/+*
w; ap^{MD544}/CyOen11	wing discs	*w; ap^{MD544}/+;* *P{UAS-EGFP}34/+*	eGFP	*w; ap^{MD544}/ P{UAS-Avic/GFP.* *dsRNA.R}143; P{UAS-EGFP}34/+*
w; pxn-Gal4	hemocytes	*w; P{UAS-GFP.S65T}T2/+;* *pxn-Gal4/+*	GFP	N/A
w; FRT-G13, tubP-Gal80;tubP- *Gal4/TM6B, Tb*	none	*w; FRT-G13, tubP-Gal80/P{UAS-* *GFP.S65T}T2;tubP-Gal4/+.*	Gal80	N/A

The list of Gal4 lines used in this study. Genotypes of crosses and the EGFP (or GFP) expression pattern in the last larval stage are also listed. EGFP was used for most of the experiments. GFPS65T was used for monitoring reporter expression in hemocytes (pxn-Gal4), because we could not detect a strong EGFP signal in the hemocytes.

Figure 1

Larval RNAi in *Drosophila* reporter lines. **(A-F; Left)** EGFP expression driven by various Gal4 lines. **(A-F; Middle)** EGFP expression 24 hours after injection of *EGFP* dsRNA. **(A-F; Right)** EGFP expression in *EGFP* hairpin RNA co-expressing flies. All *Drosophila* larvae are last larval instar, oriented anterior left, posterior right.
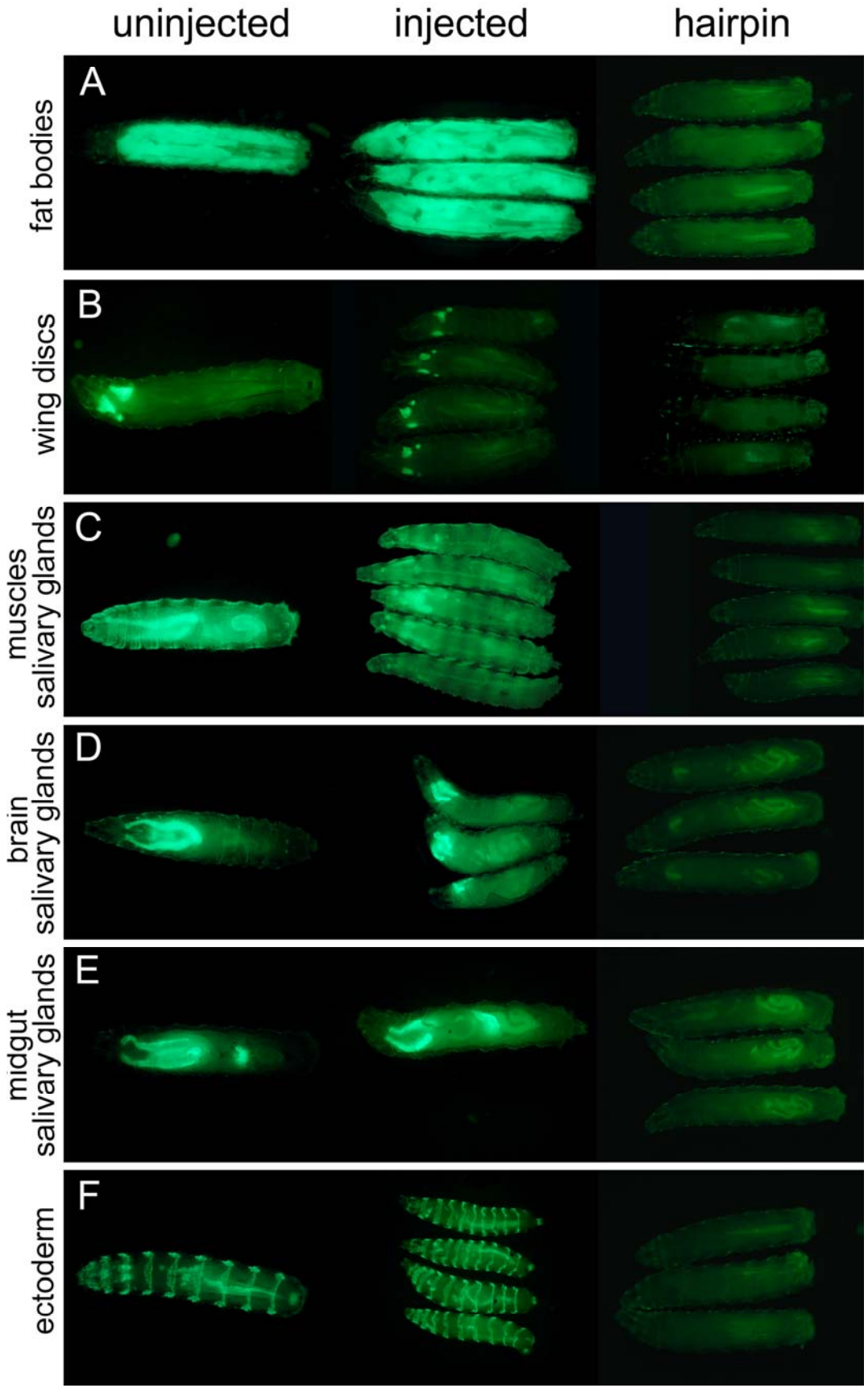
83

Figure 2

Larval RNAi in *Drosophila* hemocyte line. (**A; Left and Middle)** GFP expression in the

hemocytes driven by *pxn-Gal4*. (**A; Right)** GFP expression 24 hours after injection of *GFP*

dsRNA. (**B; Left and Middle**) Pupal stage GFP expression in hemocytes driven by *pxn-Gal4*.

(**B; Right)** Pupal stage GFP expression 48 hours after injection of *GFP* dsRNA into larvae. All

Drosophila larvae are last larval instar. Larvae and pupae are oriented anterior left, posterior
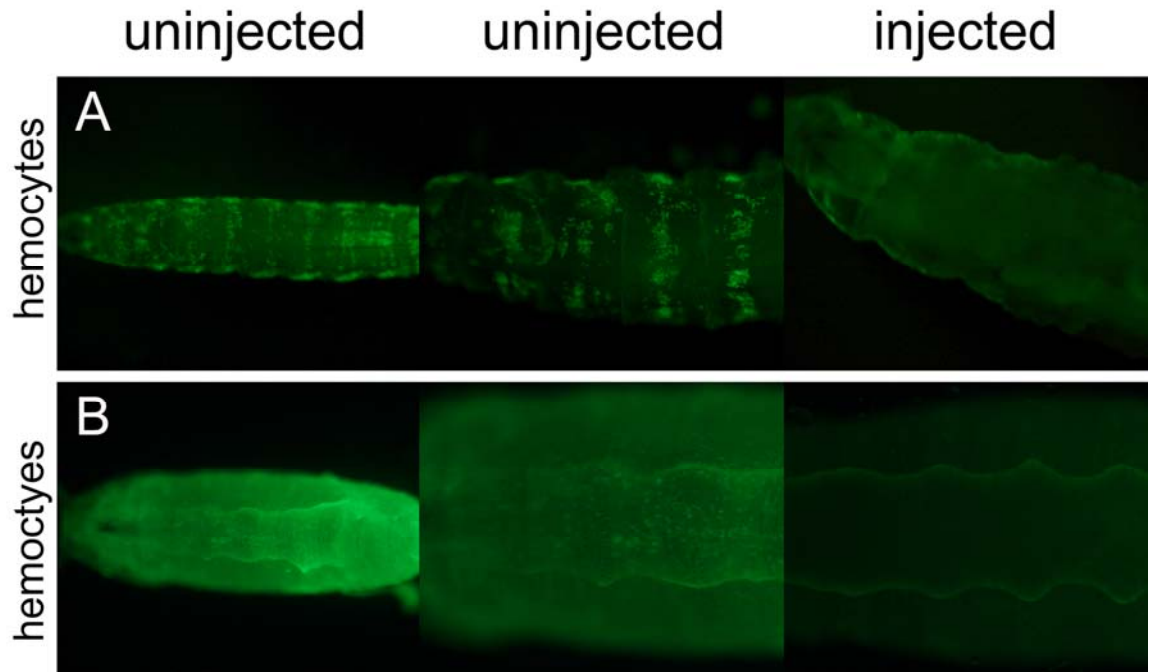
right.

uninjected   uninjected   injected

A

hemocytes

B

hemoctyes

Figure 3

Injection of *Gal80* dsRNA in *Drosophila* larvae. **(A)** *Gal4/UAS* and the *Gal80/Gal4/UAS* system. (**Left)** The ubiquitous expression of Gal4 drives GFP expression in all tissues (*tub-Gal4 UAS-GFP*). (**Middle)** Gal80 represses the function of Gal4 resulting in individuals with no GFP expression (*tub-Gal80/Gal4 UAS-GFP*). (**Right)** GFP expression is only seen in tissues that take up the *Gal80* dsRNA and mount an RNAi response (*tub-Gal80/Gal4 UAS-GFP* injected with *Gal80* dsRNA). (**B; Left)** Larval epidermis and adult with GFP expression in the hemocytes driven by *pxn-Gal4*. (**B; Middle)** *tub-Gal80/Gal4 UAS-GFP* larval cuticle and adult. No GFP expression is observed due to repression by Gal80. (**B; Right)** *tub-Gal80/Gal4 UAS-GFP* larval epidermis and adult after larval injection of *Gal80* dsRNA. Arrows point to GFP expressing hemocytes. Arrowhead indicates injection wound.
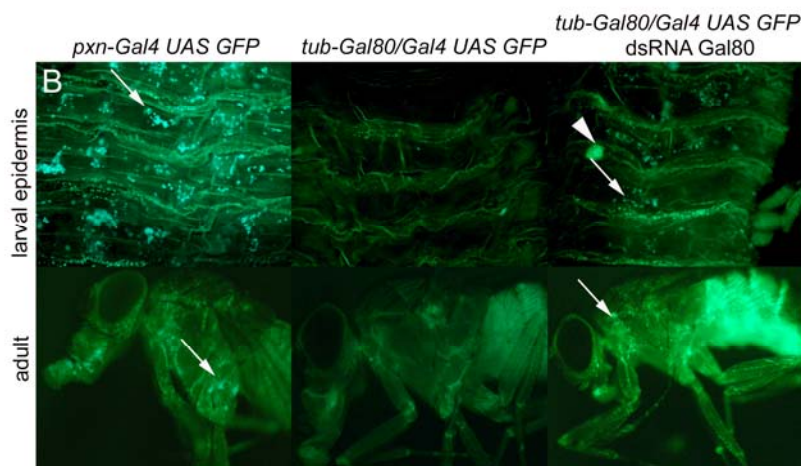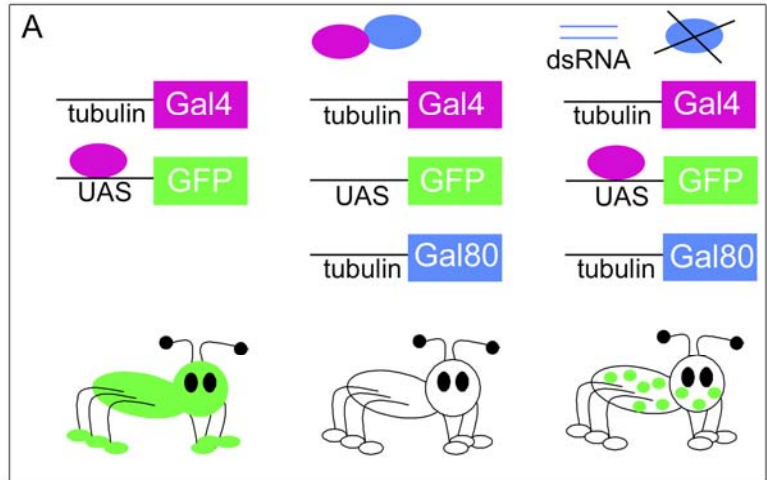
Figure 4

Injection of *EGFP* dsRNA in *Tribolium* larvae**.** **(A)** Lateral view of *aTub-EGFP Tribolium* larvae. Upper larva was uninjected. Lower two larvae were injected with *EGFP* dsRNA. **(B)** Lateral view of *aTub-EGFP Tribolium* larva injected with *EGFP* dsRNA. **(C)** Ventral view of *aTub-EGFP Tribolium* larvae. Upper larva was uninjected. Lower two larvae were injected with *EGFP* dsRNA. **(D)** Ventral view of *aTub-EGFP Tribolium* larvae injected with *EGFP* dsRNA. **(E)** *aTub-EGFP Tribolium* pupa.   **(F)** *aTub-EGFP Tribolium* pupa injected with *EGFP* dsRNA. *Tribolium* larvae are oriented anterior left, posterior right. EGFP expression was documented 48 hours after injection of dsRNA. Pupae are shown ventral view, oriented anterior up, posterior down. EGFP expression was documented 96 hours after injection of dsRNA. Arrows point to residual EGFP expression.
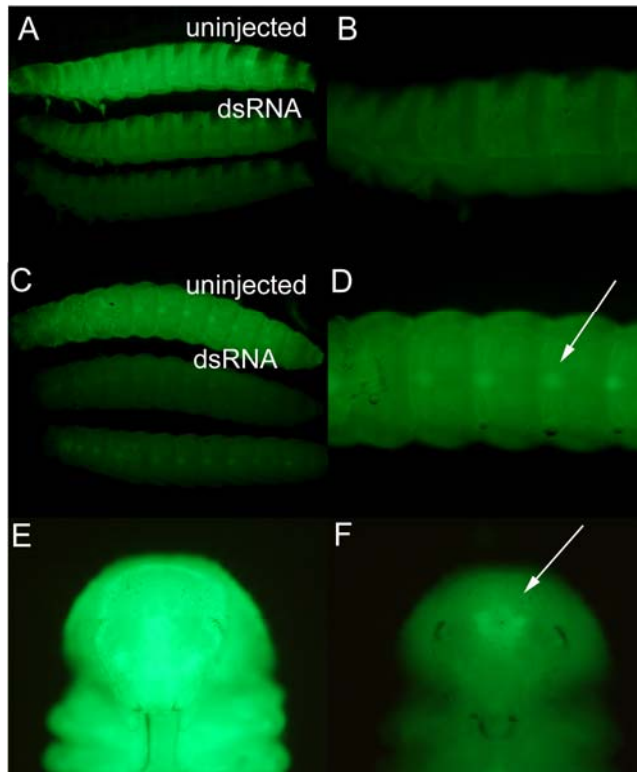
Chapter III

Survey of RNAi component genes in the beetle *Tribolium castaneum*

Sherry C. Miller
Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506

The concepts and the majority of the data in this chapter were published in <u>Genome Biology</u> in January 2008. The paper was entitled "Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*". The functional data in this paper were provided by me and I participated in the discussion, organization, and editing of the paper. Because I was not involved in writing the initial paper I have written this chapter in order to include my contributions to the paper in my dissertation.

Abstract

RNA interference (RNAi) is a highly conserved cellular mechanism of gene silencing triggered by double-stranded (dsRNA). Some organisms, including the nematode *Caenorhabditis elegans*, have a systemic RNAi response, in which the response occurs throughout the organism regardless of the site of dsRNA entry. Recently many insects, including *Tribolium castaneum*, have also been shown to have a robust systemic RNAi response. However, the leading insect model *Drosophila melanogaster* does not. To study the systemic RNAi response in insects, we have utilized the newly sequenced *Tribolium* genome to identify and analyze *Tribolium* RNAi machinery genes and systemic RNAi candidate genes. Phylogenetic analysis suggests that *Tribolium* has a slightly larger inventory of potential RNAi machinery genes than *Drosophila* and functional analysis suggests that *Tc-Dicer-2*, *Tc-Argonauate-2a* and *Tc-Argonaute-2b* are essential for the RNAi response. Our results were unable to confirm that *Tc-R2D2* or *Tc-C3PO* (homologous to *Dm-R2D2*) are essential for the RNAi process in *Tribolium*. We also identified three *Tribolium* homologs (*Tc-silA*, *Tc-silB*, *Tc-silC*) of *C. elegans sid-1*, which encodes a transmembrane domain protein essential for the systemic RNAi response. While *Tc-silA*, *Tc-silB* and *Tc-silC* share sequence homology with *Ce-sid-1*, closer analysis reveals that the *Tribolium* homologs have more identity with the *sid-1* related gene *Ce-tag-130*. Furthermore, functional data of *Tc-silA*, *Tc-silB* and *Tc-silC* indicates that they are not involved in the systemic RNAi response in *Tribolium* suggesting that they are not true orthologs of *Ce-sid-1*. Although both *Tribolium* and *C. elegans* possess a robust systemic RNAi response, our data suggests that the method of dsRNA uptake is not conserved. Further functional analysis of systemic RNAi candidate genes needs to be performed in order to

completely understand the degree of conservation of the systemic RNAi response and to fully

utilize RNAi as a genetic tool.

Introduction

RNA interference

RNA interference (RNAi) is a mechanism of gene regulation that has been shown to be widely conserved across both plant and animal phyla. The interference mechanism is triggered when double-stranded RNA (dsRNA) that is homologous to endogenous message enters a cell (or is transcribed within a cell) (Fire et al. 1998; Meister and Tuschl 2004; Mello and Conte 2004). Once in the cell the dsRNA is bound by a complex of proteins including the RNase III endonuclease Dicer (Dcr), which cleaves the dsRNA into small dsRNA fragments termed small interfering RNAs (siRNA) (reviewed in Carmell and Hannon 2004). siRNAs are then loaded into RNA induced silencing complexes (RISC) (reviewed in Filipowicz 2005) with the help of dsRNA binding motif (dsRBM) proteins such as R2D2 and Loquacious (Loqs) (Tabara et al. 2002; Liu et al. 2003; Pellino and Sontheimer 2003; Forstemann et al. 2005; Leuschner et al. 2005; Saito et al. 2005). Once in the complex siRNAs are used as a guide to find target mRNAs, which are then silenced by Argonaute (Ago) family protein-mediated cleavage (reviewed in Carmell et al. 2002; Parker and Barford 2006).

In addition to the RNAi pathway, recent studies have highlighted the importance of a related pathway termed the microRNA (miRNA) pathway. While the miRNA pathway is not the focus of this research, it is relevant because it uses related (and sometimes identical) proteins. The miRNA pathway is found in most eukaryotic organisms and is essential for negative gene regulation in such processes as growth, development, differentiation and metabolism (Niwa and Slack 2007). miRNA precursors are synthesized within a cell by RNA polymerase II and processed into mature miRNAs by two RNase III enzymes, Drosha and Dicer. Then like siRNAs, miRNAs are incorporated into silencing complexes, which include Argonaute family

proteins. After incorporation into the silencing complex, miRNAs bind the 3' UTR of their targets and regulate them through translational repression or cleavage (reviewed in Pasquinelli et al. 2005; Ouellet et al. 2006).

Systemic RNAi

The first animal in which RNAi was described, and has since been studied in extensive detail, is the model nematode *C. elegans*. Early work in *C. elegans* RNAi revealed that the site of dsRNA injection did not impact the resulting phenotype, suggesting that a gene-specific silencing signal was being transported between cells (Fire et al. 1998; May and Plasterk 2005; Hunter et al. 2006). In *C. elegans* this phenomenon, termed systemic RNAi, has since been shown to involve two steps; dsRNA uptake from the extracellular environment and subsequent spreading of dsRNA from cell to cell (Timmons et al. 2003; Tomoyasu et al. 2008). Continued work on systemic RNAi in *C. elegans* illustrated that systemic RNAi could be triggered by providing dsRNA to worms via soaking or feeding (Tabara et al. 1998; Timmons et al. 2001).

The ability to feed dsRNA to *C. elegans* has allowed several large-scale mutant screens for genes involved in systemic RNAi, and a number of genes that are essential for the uptake of dsRNA have been identified (Winston et al. 2002; Tijsterman et al. 2004). These genes are referred to as systemic RNAi defective (sid) (Winston et al. 2002) or RNAi spreading defective (rsd) genes (Tijsterman et al. 2004). The proteins encoded by five of the genes identified in these screens have been characterized. SID-1, a multipass transmembrane protein, is involved in the uptake and spreading of dsRNA in both somatic and germ line cells (Winston et al. 2002; Feinberg and Hunter 2003). SID-2, a transmembrane protein expressed on the apical side of gut cells, is involved in taking up dsRNA into the gut cells after dsRNA feeding (but is not involved in further spreading of the dsRNA) (Winston et al. 2007). Three other genes, *rsd-2*, *rsd-3*, and

rsd-6, have been identified as having a role in dsRNA uptake into the germ line (Tijsterman et al. 2004).

Recently, systemic RNAi-like responses, in which dsRNA delivery elicits an RNAi response in tissues far away from the injection site, have been reported in a variety of insects (Marie et al. 2000; Nishikawa and Natori 2001; Bettencourt et al. 2002; Blandin et al. 2002; Rajagopal et al. 2002; Aljamali et al. 2003; Amdam et al. 2003; Zhu et al. 2003; Liu and Kaufman 2004; Narasimhan et al. 2004; Tabunoki et al. 2004; Tomoyasu and Denell 2004; Araujo et al. 2006; Cruz et al. 2006; Kuwayama et al. 2006; Lynch and Desplan 2006; Turner et al. 2006; Zhou et al. 2006; Baum et al. 2007; Ghanim et al. 2007). This type of RNAi response almost certainly involves cellular uptake of dsRNA from the hemoceol, but it does not necessarily involve spreading of dsRNA from cell to cell. Therefore, when referring to systemic RNAi in insects we are referring to the ability to achieve an RNAi response in cells away from the site of dsRNA injection rather than to a strict definition of dsRNA uptake and spreading.

The best studied insect model, *Drosophila melanogaster*, does not appear to have a robust systemic RNAi response, since injection of dsRNA into the *Drosophila* body cavity is not an effective method of dsRNA delivery (Miller et al. 2008). In contrast, we have shown that the systemic RNAi response in the red flour beetle, *Tribolium castaneum*, is very robust, as virtually all cell types are susceptible to dsRNA once it is introduced into the organism (Miller et al. 2008). Therefore, we propose to use *Tribolium* as a model to study the mechanism of systemic RNAi in insects.

For this study we asked what genetic mechanisms might be responsible for the differences leading to the lack of response in *Drosophila* and the exceptionally robust systemic RNAi response seen in *Tribolium*. We considered the following hypotheses. First, perhaps

Tribolium has more efficient RNAi machinery thereby allowing a more robust systemic response. Second, perhaps *Tribolium* cells have the ability to uptake and/or transport dsRNA efficiently, whereas *Drosophila* cells lack this ability. Our results indicate that while the *Tribolium* genome has additional RNAi machinery genes there is no evidence that these genes enhance the robustness of the systemic RNAi response. Additionally, we find no evidence that the dsRNA uptake mechanism is conserved among *Tribolium* and *C. elegans*, suggesting that insects with a systemic RNAi response uptake dsRNA through a different mechanism.

Materials and Methods

Cloning genes

Total RNA was extracted from *Tribolium* Ga-1 pupae using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). *Tribolium* homologs of the Dicer, Argonaute and dsRBM proteins were identified using reciprocal BLAST analysis of *Drosophila* homologs. *Tribolium* Sid-like homologs were identified using reciprocal BLAST analysis of Ce-SID-1. Primers were designed with the PrimerSelect module of Lasergene (DNASTAR, Inc.). PCR amplified fragments of each gene were cloned into pCR4-TOPO using the TOPO TA Cloning Kit (Invitrogen).

dsRNA synthesis

Template preparation for dsRNA synthesis was performed by PCR using a single primer designed against the pCR4-TOPO vector sequence flanking the insertion site with a T7 promoter sequence at the 5' end as described previously (Tomoyasu et al. 2008). For *EGFP* dsRNA template, gene specific forward and reverse primers with T7 promoter sequence at their 5' ends were used as described previously (Tomoyasu and Denell 2004). dsRNA was synthesized using the MEGAscript T7 High Yield Transcription kit (Ambion, Austin, TX, USA).

Larval injections

Larvae were injected as described previously (Tomoyasu and Denell 2004). dsRNA for candidate genes were injected first at a concentration of 1ug/ul (approximately 0.5 ug/larva). dsRNA for *EGFP* was injected 48 hours later at a concentration of 0.01ug/ul (approximately 0.005 ug/larva).

Documentation

Larvae were analyzed for EGFP expression 72 hours after the second injection and pupae were analyzed 1 week after the second injection. Larvae and pupae were documented using an Olympus SZX12 microscope and a Nikon DXM 1200F digital camera. Identical exposure times were used for each image.

Results

To test the involvement of *Tribolium* genes in the RNAi pathway, we devised an *in vivo* assay system utilizing a transgenic line of beetles (Pu 11) expressing EGFP in the eyes and future wing primordia (Tomoyasu and Denell 2004). Our assay system is composed of two sequential injections. Initially, we inject dsRNA for the putative RNAi component gene. Then, two days later, dsRNA corresponding to EGFP is injected. If the candidate gene plays an essential role in RNAi, removing its function should inhibit the ability of the organism to perform RNAi when *EGFP* dsRNA is introduced, meaning that EGFP will still be expressed (Fig 1A). If the candidate gene is not essential for the RNAi response, then its loss of function should not affect the later RNAi and EGFP expression will be knocked down (Fig 1A). Controls for this assay system were performed, in which genes not involved in the RNAi pathway (*Ultrabithorax* and *dsRed*) were knocked down, followed by *EGFP* RNAi. *dsRed* represents an exogenous gene with no mRNA target in *Tribolium* (Fig 1D). *Ubx* is a control for an endogenous gene, as it is expressed in the *Tribolium* hindwing (Tomoyasu et al. 2005) (Fig 1E). When either *dsRed* or *Ubx* dsRNA was injected followed by *EGFP* dsRNA EGFP expression was not seen indicating that, at the concentrations chosen (1ug/ul *dsRed* or *Ubx* dsRNA and 0.01ug/ul *EGFP* dsRNA), the initial dsRNA does not inhibit the ability of the *EGFP* dsRNA to knock down *EGFP* (Fig 1B-E).

Core Machinery

To test the hypothesis that the efficient systemic RNAi response of *Tribolium* results from particularly effective core RNAi machinery, we performed functional analysis of *Tribolium* homologs of the Dicer, Argonaute and dsRBM proteins.

Dicer

In *C. elegans* there is one Dicer protein that acts in both the RNAi and the miRNA pathway (Bernstein et al. 2001; Ketting et al. 2001; Knight and Bass 2001). In *Drosophila* there are two Dicers, Dm-Dcr-1 and Dm-Dcr-2, which function in the miRNA pathway and the RNAi pathway, respectively (Lee et al. 2004). *Tribolium* also has two Dicer proteins (Tomoyasu et al. 2008) (Table 1). Tc-Dcr-1 is an ortholog of Dm-Dcr-1 and homologous to Ce-DCR-1. However, phylogenetic analysis does not show that Tc-Dcr-2 is a clear ortholog of Dm-Dcr-2, although it shares similar domain architecture. Furthermore, the domain architecture of Tc-Dcr-1 is more similar to Ce-DCR-1 than to Dm-Dcr-1 (Tomoyasu et al. 2008), suggesting that perhaps both *Tribolium* Dicers have the ability to act in the RNAi pathway. We hypothesized that having two Dicers with the potential to function in the RNAi pathway may contribute to the robustness of the RNAi response in *Tribolium*.

When transgenic *Tribolium* larvae were first injected with *Tc-Dcr-1* dsRNA and then later injected with *EGFP* dsRNA, we saw an absence of EGFP expression (Fig 2A), suggesting that Tc-Dcr-1 is not essential for the RNAi pathway. When the same experiment was performed using *Tc-Dcr-2,* we saw EGFP expression in 10 of 17 (~59%) experimental individuals (Fig 2B), indicating that by knocking down Tc-Dcr-2 function we were able to inhibit the RNAi response. While our data suggest that Tc-Dcr-2 is the only Dicer essential for RNAi, it is possible that Tc-Dcr-1 may play a role in RNAi but its absence is compensated by Tc-Dcr-2. In order to test this hypothesis we performed a double knockdown of Tc-Dcr-1 and 2 followed by injection of *EGFP* dsRNA. In this experiment, 12 of 30 (40%) experimental individuals showed EGFP expression (Fig 2C). The fact that the double RNAi did not enhance the effect of Tc-Dcr-2 RNAi indicates that despite the domain architecture of Tc-Dcr-1, it is unlikely to be involved in the RNAi pathway.

dsRNA binding motif proteins (dsRBM)

In *Drosophila*, dsRBM proteins act with RNase III endonucleases to load small RNA molecules into the silencing complexes (Tabara et al. 2002; Pellino and Sontheimer 2003; Forstemann et al. 2005; Leuschner et al. 2005; Saito et al. 2005). Pasha is the dsRBM protein that interacts with Drosha (Denli et al. 2004) and Loqs is the dsRBM protein that acts with Dcr-1 (Forstemann et al. 2005; Leuschner et al. 2005; Saito et al. 2005). Both of these dsRBM proteins are important in the miRNA pathway. R2D2 is the dsRBM protein that acts with Dcr-2 in the RNAi pathway (Liu et al. 2003; Pellino and Sontheimer 2003). It has been hypothesized that these dsRBM proteins determine the specificity of Dicer proteins in *Drosophila* (Leuschner et al. 2005; Saito et al. 2005). If this is true perhaps the number and type of dsRBM proteins affect the efficiency of the RNAi pathway. In *Tribolium* there are clear one to one orthologs of *Drosophila loqs* and *pasha*. However, there are two genes that show some similarity with *R2D2*, named *Tc-R2D2* and *Tc-C3PO* (Tomoyasu et al. 2008) (Table 1).

We hypothesized that having two dsRBM proteins capable of acting in the RNAi pathway may increase *Tribolium's* RNAi response. To test this hypothesis we removed the function of R2D2 and C3PO by RNAi. dsRNA for *R2D2*, *C3PO*, or a mixture of both was injected into *Tribolium* larvae, followed by the second injection of dsRNA for *EGFP*. Surprisingly, we found that removing the function of these dsRBM proteins had no effect on *Tribolium's* ability to perform RNAi, as EGFP was efficiently knocked down in all the experimental groups (Fig 3). We therefore hypothesized that perhaps the other related dsRBM protein known to interact with Dcr proteins, Loqs, is not specific to the miRNA pathway and may be able to compensate for the loss of R2D2 and C3PO. We tested this hypothesis by removing the function of Loqs alone and removing the function of all three dsRBM proteins.

Again, we found that knocking down the dsRBM proteins had no effect on subsequent RNAi (Fig 4). These data suggest that the dsRBM proteins may not be essential for the RNAi response in *Tribolium*.

Argonaute

Argonaute proteins are essential components of silencing complexes, and as such play an important role in both the RNAi and miRNA pathways. Different Argonaute proteins are involved in transcriptional silencing, siRNA post-transcriptional silencing and miRNA post-transcirptional silencing (Carmell et al. 2002; Filipowicz 2005; Parker and Barford 2006). In *Drosophila*, Ago-1 is used in the miRNA pathway, Ago-2 is essential in the RNAi pathway (Okamura et al. 2004), and Piwi, Aubergine and Ago-3 are important in transcriptional silencing (Pal-Bhadra et al. 2004; Brennecke et al. 2007; Lin 2007). In *C. elegans* there is a massive expansion of the Argonaute proteins (27 identified) and as in *Drosophila* these proteins function in different processes (Grishok et al. 2001; Tabara et al. 2002; Yigit et al. 2006). Some Argonautes are involved in the miRNA pathway while others are involved in the RNAi pathway. One important class of Argonaute proteins in *C. elegans* is the secondary Argonautes, which interact with siRNAs that have been amplified via RNA dependent RNA Polymerases (RdRPs). The action of these secondary Argonautes is an essential, rate-limiting step of the RNAi response in *C. elegans* (Yigit et al. 2006). Like *Drosophila*, *Tribolium* has five *Argonaute* genes. However, there is not a one to one correspondence with the *Drosophila genes*. There is a single *Tribolium* ortholog of *Dm-Ago-1*, but two *Tribolium Ago-2* paralogs (*Tc-Ago-2a* and *b*) that appear to be the result of a lineage specific duplication. There is only one *Tribolium* ortholog corresponding to the Piwi/Aub class of Argonautes, as well as a single ortholog to *Dm-Ago-3* (Tomoyasu et al. 2008) (Table 1).

Since Ago-2 is important for the RNAi pathway in *Drosophila*, we hypothesized that the presence of two Ago2 proteins in *Tribolium* may contribute to the robust systemic RNAi response of this beetle. To test this hypothesis we performed RNAi on *Tc-Ago-1*, *Tc-Ago-2a*, or *Tc-Ago-2b*, individually as well as on *Tc-Ago-2a* and *2b* concurrently. After the second injection of dsRNA for *EGFP* we examined the individuals for EGFP expression. We found that removing Tc-Ago-1 had no effect on subsequent RNAi, as EGFP was efficiently knocked down (Fig 5A). While the function of the RNAi pathway did not appear to be affected by *Tc-Ago-1* RNAi, we did see a phenotype that suggests a role for Tc-Ago-1 in the miRNA pathway. When *Tc-Ago-1* RNAi was performed, the injected individuals were incapable of pupation. As the miRNA pathway has been shown to play a role in both *C. elegans* developmental timing (Feinbaum and Ambros 1999; Reinhart et al. 2000; Grishok et al. 2001) and insect metamorphosis (Bashirullah et al. 2003), these data are consistent with a role of Tc-Ago-1 in the miRNA pathway. In contrast to the Tc-Ago-1 results, we found that removing the function of either of the *Tc-Ago-2* genes resulted in a decrease in RNAi efficiency (Fig 5B, C). 8 of 28 (~29%) *Tc-Ago-2a* RNAi individuals and 12 of 28 (~43%) *Tc-Ago-2b* individuals showed EGFP expression. Our data indicate that while both *Tc-Ago-2* genes are involved in the RNAi pathway they are not redundant, as an effect can be seen by the removal of just one homolog. Interestingly, double RNAi of *Tc-Ago2a* and *2b* does not enhance the effect (Fig 5D) (12 of 44; ~27%). The results indicate that *Tribolium* has duplicated *Argonaute* genes that are functional in the RNAi pathway but our data does not necessarily indicate that *Tribolium's* RNAi response is enhanced due to the duplication.

Uptake

Sid-1

We next addressed the hypothesis that the robust systemic RNAi response of *Tribolium* is due to efficient uptake and/or transport of dsRNA. Several genes have been identified as playing a role in systemic dsRNA uptake in *C. elegans* (Winston et al. 2002; Feinberg and Hunter 2003; Tijsterman et al. 2004; Winston et al. 2007). The transmembrane protein SID-1 is the best studied and plays a role in the widest variety of tissue, as it is important for dsRNA uptake in both somatic and germ line cells (Winston et al. 2002; Feinberg and Hunter 2003). *sid-1* homologs have been identified in a variety of species (Winston et al. 2002; Duxbury et al. 2005; Aronstein 2006), although their function has remained largely unexplored. Interestingly, *Drosophila* does not have a *sid-1* homolog, leading to speculation that its absence is the reason for *Drosophila's* lack of a systemic RNAi response (Dong and Friedrich 2005; Duxbury et al. 2005; Aronstein 2006; Consortium 2006). Additional work in *Drosophila* indicates that overexpression of *Ce-sid-1* in culture cells increases the ability of these cells to uptake dsRNA from the external environment further supporting the idea that *sid-1* is important in dsRNA uptake (Feinberg and Hunter 2003).

In contrast to *Drosophila*, *sid-like* genes have been identified in several other insect species including *Tribolium* (Tomoyasu et al. 2008). In *Tribolium* three *sid-like* genes have been identified (*silA-C*) Unfortunately, phylogenetic analysis has been unable to resolve the relationship between insect *sil* genes and *C. elegans' sid-1* (Tomoyasu et al. 2008). The N-terminal region of SID-1 is the extracellular domain while the C-terminal region is composed of transmembrane domains. The C-terminal transmembrane domain is believed to act as a channel for dsRNA molecules (Winston et al. 2002; Feinberg and Hunter 2003). When the C-terminal region of the protein is used for phylogenetic analysis, the relationship between *sil* genes, *sid-1* and the related *C. elegans* gene *tag-130* is unresolved. However, if the amino terminal region of

the protein is compared it appears that the *Tribolium sil* genes, as well as other insect and vertebrate *sid-like* genes, are much more similar to *tag-130* than *sid-1* (Tomoyasu et al. 2008). While the function of TAG-130 in *C. elegans* is unknown, we showed that it does not play a role in dsRNA uptake (Tomoyasu et al. 2008). The inconclusive relationship between *sid-like* genes and *sid-1* raises the question of whether *sil* genes in *Tribolium* play any role in dsRNA uptake.

As stated previously there are three *sid-like* genes in *Tribolium*, *silA*, *silB* and *silC*. Our functional analysis indicates that performing RNAi for each of the *sil* genes independently, or performing a triple RNAi has no effect on the individual's ability to perform subsequent RNAi (as EGFP expression is always absent after *EGFP* RNAi) (Fig 6A-D). These results suggest that the *Tribolium sil* genes do not play a role in *Tribolium's* systemic RNAi response. However, these results are not conclusive and must be interpreted with care. Performing triple RNAi is a difficult procedure as competition between dsRNAs is known to occur which can result in a decrease in the effectiveness of the RNAi response (Castanotto et al. 2007; Yoo et al. 2008). Therefore, the possibility remains that *sil* genes play a redundant role in *Tribolium's* systemic RNAi response and we were unable to efficiently remove the function of all *sil* genes during a triple RNAi experiment.

Discussion

While work in *C. elegans* has provided invaluable data for the uptake and function of dsRNA there has been very little work done in other organisms. The work described here is the first look at the function of RNAi component genes in an emerging model system. If the mechanism of systemic RNAi is conserved between nematodes and insects we would expect to see conservation of RNAi components between *C. elegans* and *Tribolium*. In contrast, we might expect at least some of these components to be absent in *Drosophila*.

Core Machinery Genes

There are several differences between the RNAi core machinery genes in *Drosophila* and *Tribolium*. The first is that the Tc-Dcr-1 protein has a domain architecture more similar to Ce-DCR-1 (Tomoyasu et al. 2008) suggesting that Tc-Dcr-1 may be involved in both the miRNA and the RNAi pathway. Results from *Dcr* RNAi experiments indicate that, as in *Drosophila*, Dcr-2 is involved in the RNAi pathway. In contrast, we did not find any indication that Tc-Dcr-1 is involved in the RNAi pathway. These data suggest that the two Dcr genes in *Tribolium* may function in a similar way as in *Drosophila*, with Dcr-1 involved in the miRNA pathway and Dcr-2 involved in the RNAi pathway. However, we did not detect phenotypes in either the *Tc-Dcr-1* or the double *Dcr* RNAi that would suggest Dcr-1's involvement in the miRNA pathway. In fact, with the exception of three individuals showing possible wing expansion defects we did not find any phenotype associated with Tc-Dcr-1 knockdown. One explanation for this lack of phenotype is that removing the miRNA pathway at this life stage in *Tribolium* does not result in any observable phenotype. We find this explanation unlikely as knockdown of Tc-Ago-1 does result in a metamorphosis phenotype. Another possibility for this lack of phenotype is that Tc-Dcr-1 is not essential for the miRNA pathway in *Tribolium*. Perhaps another protein can

compensate for the loss of Dcr. If this is the case it is possible that Tc-Dcr-1 can act in the RNAi pathway as well but the compensating protein masks its role. The final possibility is that Tc-Dcr-1 is essential for the miRNA pathway but we were unable to completely remove its function by RNAi.

The second difference between the RNAi core machinery genes in *Drosophila* and *Tribolium* is that in *Tribolium* there are two dsRBM proteins that maybe involved in the RNAi pathway, whereas *Drosophila* only has one (Tomoyasu et al. 2008). Removing any or all of the dsRBM proteins, R2D2, C3PO and Loqs did not appear to affect the ability of *Tribolium* to perform RNAi at a later date. This is surprising since work in *Drosophila* has shown that R2D2 is essential for the RNAi effect to occur. In *Drosophila* R2D2 mutant embryos, injected dsRNA has no effect (Liu et al. 2003). This is presumably due to R2D2's important role in Dicer-2 stability and its role in siRNA loading into RISC. Again several explanations are possible for these results. First, there is another dsRBM protein identified in *Drosophila* called Pasha. Pasha is known to be involved in miRNA pathway through interactions with its partner Drosha (Denli et al. 2004). While there is no evidence of Pasha being involved in the RNAi pathway it is possible that it can compensate for the loss of the other dsRBM proteins. Second, removing R2D2, C3PO and Loqs requires triple RNAi, which may result in competition between the three dsRNAs. This competition may decrease the effectiveness of the RNAi resulting in incomplete knock down. Finally, the dsRBM proteins may not be absolutely essential to the RNAi pathway in *Tribolium*. Perhaps their RNase III enzyme partners can function alone.

It is also important to note that RNAi of *loqs* did not affect pupation or cause any other obvious morphological phenotypes. This indicates that in addition to not affecting the RNAi pathway *loqs* RNAi also did not disrupt the miRNA pathway.

The final major difference between the RNAi core machinery genes in *Drosophila* and *Tribolium* is that within the Ago-2 family there appears to have been a duplication in the lineage leading to *Tribolium* (Tomoyasu et al. 2008). This duplication resulted in two Argonaute proteins, both of which may act in the RNAi pathway. Our data indicate that these two Argonuates are, in fact, essential for the RNAi pathway, as removing the function of either inhibits the RNAi response. Whether this duplication is responsible for the development of *Tribolium's* exceptionally robust RNAi response is unknown. However, this observation is intriguing because it has already been shown in *C. elegans* that Argonaute protein availability can influence the effectiveness of the RNAi response.

Other Machinery Genes

In addition to the core RNAi machinery genes there are several other RNAi component genes that may play a role in *Tribolium's* robust systemic RNAi response. For instance, in *C. elegans*, an essential step of the RNAi pathway is amplification of the dsRNA by RdRPs (Smardon et al. 2000; Sijen et al. 2001; Sijen et al. 2007). It is possible that *Tribolium* also has an efficient amplification mechanism whereas *Drosophila* does not. However, genome surveys find no evidence of RdRP-related genes in any metazoans (Tomoyasu et al. 2008), with the exception of several *Caenorhabditis* species, one mite (Gordon and Waterhouse 2007) and one cephalochordate *Branchiostoma floridae* (Vienne et al. 2003). Therefore, if an amplification mechanism exists in *Tribolium* it differs from the system in *C. elegans*.

Another type of protein that plays an important role in cell susceptiblity to RNAi in *C. elegans* is ERI-1 (Kennedy et al. 2004). ERI-1 is a nuclease responsible for siRNA degradation. In *C. elegans*, it is believed that the expression of ERI-1 causes tissues, such as nervous tissue, to be insensitive to RNAi (Kennedy et al. 2004). It is possible that overexpression of these types of

proteins in *Drosophila* may lead to the RNAi disparity between *Tribolium* and *Drosophila*. Again genome surveys are unable to provide evidence to support this hypothesis as *eri-1* like nucleases in *Tribolium* and *Drosophila* belong to a different subclass than the *Ce-eri-1* (Tomoyasu et al. 2008). The function of the *Drosophila* Eri-l like protein (Dm-Snipper) has been examined and there is no evidence that this class of Eri-1 like proteins is involved in RNAi (Kupsco et al. 2006).

dsRNA Uptake

While our functional data support the idea that the *Tc-sil* genes may not be involved dsRNA uptake, there are other systemic RNAi genes from *C. elegans* that could be considered candidate genes for uptake in *Tribolium* (Tijsterman et al. 2004; Winston et al. 2007). Unfortunately, the only other *C. elegans* systemic RNAi gene for which *Tribolium* has a homolog is *rsd-3* (*Tc-epsin-like*) (Tomoyasu et al. 2008). *Drosophila* also has an *rsd-3* homolog (*Dm-epsin-like*), even though it does not have a robust systemic RNAi response. Therefore, it is not the presence or absence of *epsin-like* that is responsible for the disparity between these two insects. It is possible, however, that expression differences of Epsin-like in *Tribolium* and *Drosophila* lead to differences in their RNAi abilities.

In addition to work done in *C. elegans,* there has been some data obtained from *Drosophila* S2 cells concerning the uptake of dsRNA (Saleh et al. 2006; Ulvila et al. 2006). While *Drosophila* appears to lack a robust systemic RNAi response, S2 cells are able to uptake dsRNA from the extracellular environment. Therefore, several RNAi screens have been done to determine what genes are necessary for this process. The 28 genes that have been identified include endocytosis genes, genes involved in intracellular transport, scavenger receptors and

genes of unknown function (Saleh et al. 2006; Ulvila et al. 2006). Whether these genes play a role in cellular uptake of dsRNA in other insect tissues remains to be seen.

System Caveats

The assay system used in this work was designed to identify whether candidate genes are involved in the RNAi pathway in *Tribolium*. However, the results must be interpreted cautiously as both false negatives and false positives are possible. If the initial candidate gene is involved in the RNAi effect but is not efficiently knocked down, false negatives can occur. If the initial candidate gene is not involved in the RNAi response but competition occurs between the candidate gene and *EGFP* dsRNA false positives could result.

We took several approaches and performed several controls to circumvent these problems. First, the dsRNA for the candidate gene was injected at a moderately high concentration (1ug/ul) in order to effectively remove its function without saturating the machinery. Second, the dsRNA for *EGFP* was used at a lower concentration (0.01ug/ul) to make the assay more sensitive to the removal of the candidate gene function. Third, the *EGFP* dsRNA was injected two days after the initial candidate gene injection to help alleviate competition that may occur at the uptake level. And fourth, controls were performed in which genes not involved in the RNAi pathway (*Ultrabithorax* and *dsRed*) were knocked down followed by *EGFP* RNAi. While there are caveats to our *in vivo* assay system, it did produce functional data that is consistent with data from other model organisms (e.g. the essential role of Dcr-2 and Ago-2 in RNAi). This assay system also produced some unexpected results in which dsRNA binding motif proteins and sid-1 like proteins do not appear essential for the RNAi response in *Tribolium*.

Conclusion

The increasing availability of genomic data and the development of techniques such as RNAi (which allows the knockdown of gene function without the use of classical genetic mutants) have allowed the study of genes, development and cellular function in a vast array of non-model systems. These types of comparative studies across phyla are essential if we hope to gain an understanding of biological conservation, diversity and evolution. However, if techniques such as RNAi are to be fully implemented in these non-model systems the mechanisms need to be understood. Our data suggest that the mechanism underlying the systemic RNAi effect seen in *C. elegans* is not conserved in *Tribolium*. Therefore, insect systemic RNAi probably employs a different method than that seen in *C. elegans*. Understanding the mechanism of systemic RNAi in *Tribolium* may allow us to render other insects amendable to RNAi so that this technique can be used for functional analysis of genes and possibly as a method of pest control.

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Table 1: Orthology of RNAi and miRNA components

	T. castaneum	*D. melanagaster*	*C. elegans*	Remarks
dsRNA bindng proteins	R2D2 C3PO	R2D2	Rde 4	
	Loquacious	Loquacious		
	Pasha	Pasha		
Bidentate RNase	Dicer 1	Dicer 1	Dicer	While Tc-Dcr 1 apears orthologous to Dm-Dcr 1 the domain architecture of Tc-Dcr-1 is more similar to that of Ce-Dcr
	Dicer 2	Dicer 2		
	Drosha	Drosha		
1ᵐArgonaute	Argonaute 1	Argonaute 1	Alg 1 Alg 2	The orthology between Prg proteins and the Ago 3 and PIWI classes of proteins is not clear
	Argonaute 2a Argonaute 2b	Argonaute 2	Rde 1 Ergo	
	Argonaute 3	Argonaute 3	Prg 1 Prg 2	
	PIWI	PIWI Aubergine		
2ᵐArgonaute			SAGO-1 SAGO-2 PPW-1 PPW2	
RdRP			Ego 1 RRF 1 RRF3	
Eri-1 like proteins			M02B7.2	
			Eri 1	
	Snipper	Snipper		
Sid 1 like proteins	Sil A Sil B Sil C		Y37H2C1 Sid 1 Tag 130	The orthology between the Tc-Sil proteins and the Ce-Sid like proteins is not resolved

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Figure 1

in vivo assay scheme for RNAi components in *Tribolium.* **(A)** Scheme of the *in vivo* assay system. **(B)** Uninjected Pu 11 larvae and pupae. EGFP expression in the wing primordial at the larval stage and in the wings and eyes at the pupal stage. **(C)** Pu 11 larvae and pupae injected with dsRNA for *EGFP* at a concentration of 0.01ug/ul. EGFP expression is absent. **(D, E)** Pu 11 larvae were injected with dsRNA for *dsRed* **(D)** or *Ubx* **(E)** at a concentration of 1ug/ul followed by dsRNA injection for *EGFP* at a concentration of 0.01ug/ul. **(F, G)** Pu 11 larvae were injected with dsRNA for *dsRed* **(F)** or *Ubx* **(G)** at a concentration of 2ug/ul followed by dsRNA injection for *EGFP* at a concentration of 0.01ugul. The injection of *dsRed* or *Ubx* dsRNA did not affect the ability of *EGFP* dsRNA to knock down EGFP expression. Individuals injected with *Ubx* dsRNA show a hindwing to elytron transformation indicating successful knockdown of Ubx function.
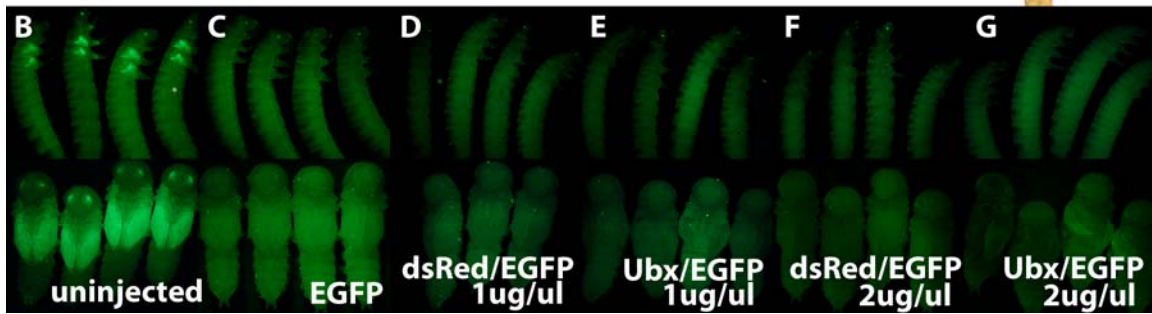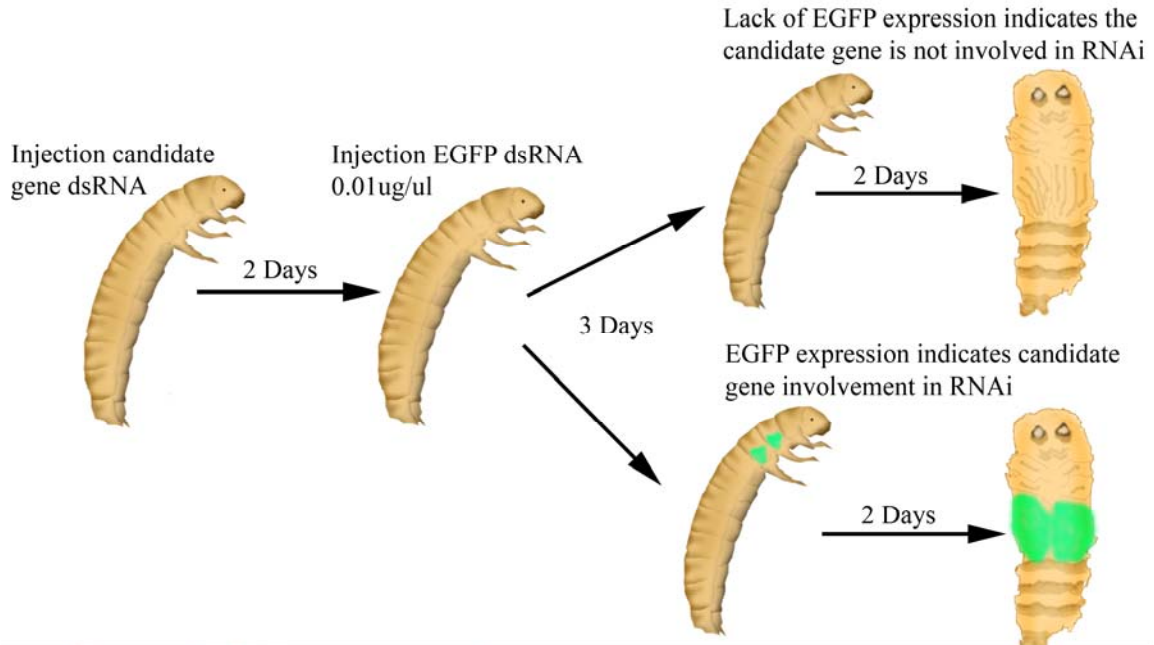
A

Injection candidate gene dsRNA

2 Days

Injection EGFP dsRNA 0.01ug/ul

3 Days

Lack of EGFP expression indicates the candidate gene is not involved in RNAi

2 Days

EGFP expression indicates candidate gene involvement in RNAi

2 Days

B uninjected **C** EGFP **D** dsRed/EGFP 1ug/ul **E** Ubx/EGFP 1ug/ul **F** dsRed/EGFP 2ug/ul **G** Ubx/EGFP 2ug/ul

Figure 2

Functional analysis of Dicer proteins in *Tribolium*. **(A)** Pu 11 larvae and pupae after injection of *Dicer-1* dsRNA followed by *EGFP* dsRNA. **(B)** Pu 11 larvae and pupae after injection of *Dicer-2* dsRNA followed by *EGFP* dsRNA. **(C)** Pu 11 larvae and pupae after coinjection of *Dicer-1* and *Dicer-2* dsRNA followed by *EGFP* dsRNA. RNAi of *Dicer-2* appears to reduce the efficiency of RNAi while RNAi of *Dicer-1* does not.
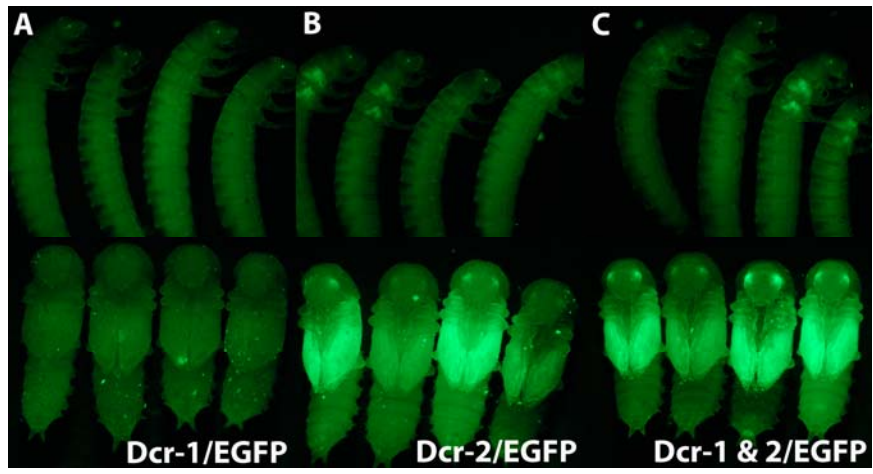
Dcr-1/EGFP
Dcr-2/EGFP
Dcr-1 & 2/EGFP

Figure 3

Functional analysis of dsRBM proteins in *Tribolium*. **(A)** Pu 11 larvae and pupae after injection of *R2D2* dsRNA followed by *EGFP* dsRNA. **(B)** Pu 11 larvae and pupae after injection of *C3PO* dsRNA followed by *EGFP* dsRNA. **(C)** Pu 11 larvae and pupae after coinjection of *R2D2* and *C3PO* dsRNA followed by *EGFP* dsRNA. RNAi of *R2D2* and *C3PO* do not appear to reduce the efficiency of RNAi.
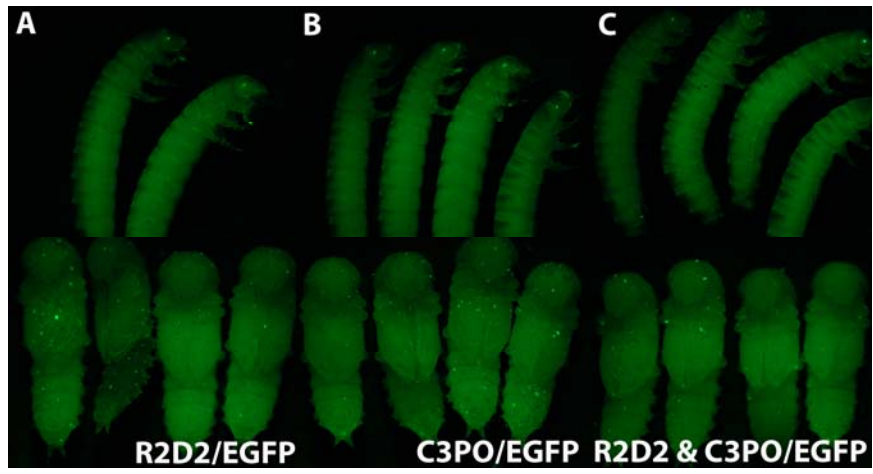
Figure 4

Functional analysis of dsRBM proteins in *Tribolium*. **(A)** Pu 11 larvae and pupae after injection of *loqs* dsRNA followed by *EGFP* dsRNA. **(B)** Pu 11 larvae and pupae after coinjeection of *loqs*, *R2D2* and *C3PO* dsRNA followed by *EGFP* dsRNA. RNAi of *loqs* does not appear to reduce the efficiency of RNAi.
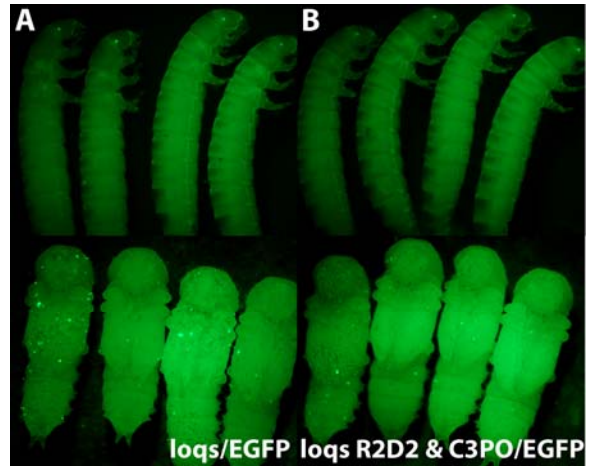
loqs/EGFP loqs R2D2 & C3PO/EGFP

Figure 5

Functional analysis of Argonaute proteins in *Tribolium*. **(A)** Pu 11 larvae after injection of *Argonaute-1* dsRNA followed by *EGFP* dsRNA. **(B)** Pu 11 larvae and pupae after injection of *Argonaute-2a* dsRNA followed by *EGFP* dsRNA. **(C)** Pu 11 larvae and pupae after injection of *Argonaute-2b* dsRNA followed by *EGFP* dsRNA. **(D)** Pu 11 larvae and pupae after coinjection of *Argonaute-2a* and *Argonaute-2b* dsRNA followed by *EGFP* dsRNA. RNAi of *Argonaute-2a* and *Argonaute-2b* appears to reduce the efficiency of RNAi while RNAi of *Argonaute-1* does not. RNAi of *Argonaute-1* results in pupation defects presumably due to interference with the miRNA pathway.
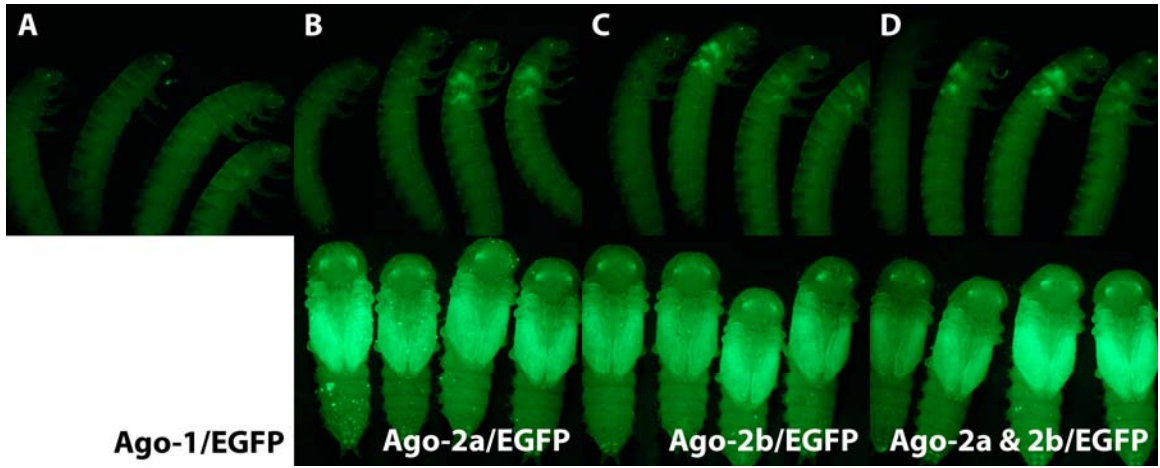
Ago-1/EGFP   Ago-2a/EGFP   Ago-2b/EGFP   Ago-2a & 2b/EGFP

Figure 6

Functional analysis of Sid-1-like proteins in *Tribolium*. **(A)** Pu 11 larvae and pupae after injection of *silA* dsRNA followed by *EGFP* dsRNA. **(B)** Pu 11 larvae and pupae after injection of *silB* dsRNA followed by *EGFP* dsRNA. **(C)** Pu 11 larvae and pupae after injection of *silC* dsRNA followed by *EGFP* dsRNA. **(D)** Pu 11 larvae and pupae after coinjection of *silA*, *silB*, and *silC* dsRNA followed by *EGFP* dsRNA. RNAi of the *sil* genes does not appear to reduce the efficiency of RNAi.

A B C D

silA/EGFP   silB/EGFP   silC/EGFP   silA B & C/EGFP
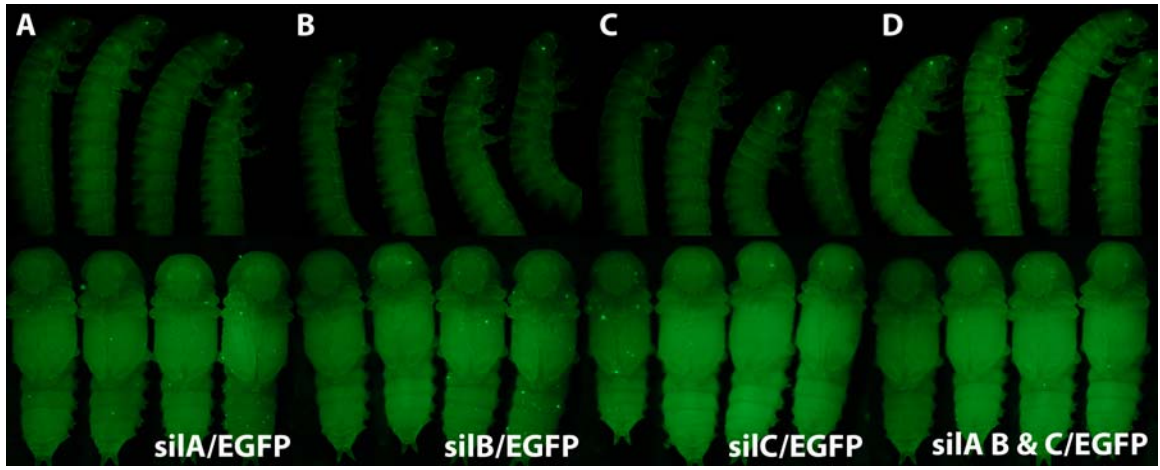
Supplementary Figure 1

Injection of dsRNA for the RNAi component genes. Control showing that injection of dsRNA

for RNAi component genes does not effect EGFP expression.
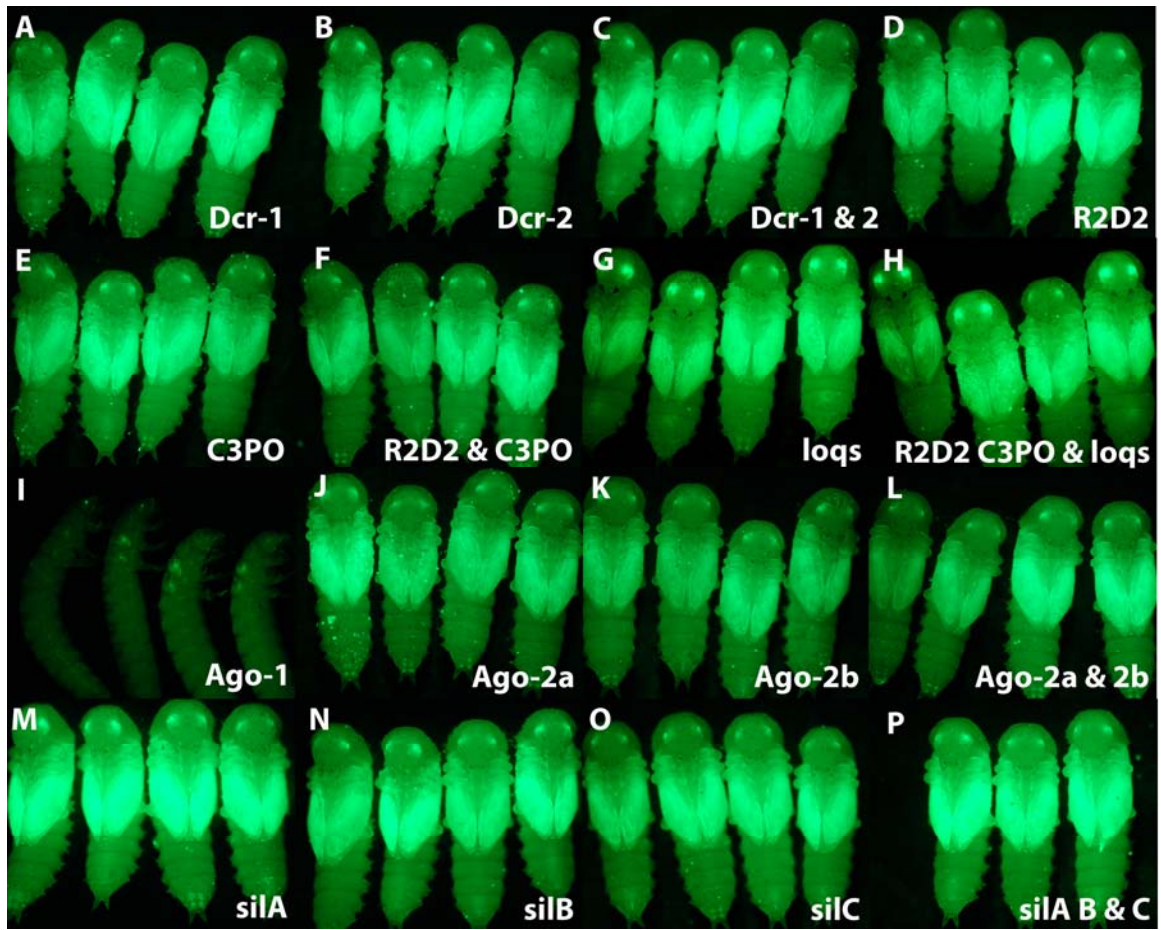
Chapter IV

Mechanisms of dsRNA uptake in the red flour beetle *Tribolium castaneum*

Running head: dsRNA uptake

Sherry C. Miller, Susan J. Brown, and Yoshinori Tomoyasu*
Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506

Keywords: RNAi, dsRNA, *Tribolium castaneum* (red flour beetle), systemic

Corresponding author: Yoshinori Tomoyasu**, Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506
**Current contact information: Department of Zoology, Miami University, 212 Pearson Hall, Oxford, OH 45056, Phone (513) 529-3162, email: tomoyay@muohio.edu

The data provided in this chapter is only present in this dissertation. At present it has not been and is not being considered for publication in any scientific journal.

Abstract

Since its discovery a decade ago, the RNA interference (RNAi) phenomenon has been identified in many eukaryotic organisms spanning both plant and animal taxonomic kingdoms. The molecular mechanism of RNAi is highly conserved. Once in a cell, double-stranded RNA (dsRNA) is processed into short interfering RNAs (siRNAs), which, upon binding with their homologous mRNAs, cause mRNA destruction resulting in a gene silencing effect. While the mechanism of RNAi appears well conserved, the method of dsRNA entry into the cells of a multiellular organism (systemic RNAi) may be less conserved. The degree to which the systemic RNAi response is conserved is unknown primarily because this process has only been extensively studied in plants and the nematode *Caenorhabditis elegans*. Furthermore, the study of systemic RNAi has been inhibited, as this response is absent in some model organisms including the fruit fly *Drosophila melanogaster*. Despite its absence in some species, recent work has established that many non-model systems do have a systemic RNAi response including many insect species. We have utilized the red flour beetle *Tribolium castaneum* as a model for dsRNA uptake in insects. Previous data suggested that the *Tribolium* homologs for the gene essential for dsRNA uptake in *C. elegans sid-1* is not involved in dsRNA uptake in *Tribolium*. Therefore, we utilized the candidate gene approach to identify other genes that may be involved in dsRNA uptake in *Tribolium*. Our data suggests that the *Tribolium* homolog of *C. elegans rsd-3* (*Tc-epsin-like*) plays an essential role in the systemic RNAi process. Our data also suggests that fucosylation may play an important role in dsRNA uptake in *Tribolium*, as the knockdown of several genes involved in this modification process resulted in a decrease in the effectiveness of RNAi. Further studies will reveal the degree to which the mechanism of systemic RNAi is conserved between organisms, which will increase the power of RNAi as a genetic tool.

Introduction

In this genomic era, RNA interference (RNAi) has emerged as a powerful reverse genetic technique to allow the study of gene function in a variety of model and non-model systems. RNAi is a gene-silencing pathway triggered by dsRNA complementary to an mRNA target (Fire et al. 1998). When "trigger" dsRNA is introduced into a cell, it is cleaved by the endonuclease Dicer into short interfering RNAs (siRNAs) approximately 21bp in length (Bernstein et al. 2001; Knight and Bass 2001; Carmell and Hannon 2004). These siRNAs are then incorporated into the RNA-induced silencing complex (RISC) (Hammond et al. 2000; Filipowicz 2005), which through the action of its catalytic component Argonaute binds the complementary mRNA target and causes its destruction (Tabara et al. 1999; Fagard et al. 2000; Hammond et al. 2001; Song et al. 2004; Parker and Barford 2006).

The mechanism of RNAi is highly conserved, with its components being found across plant and animal phyla. In many organisms, the RNAi pathway plays essential roles in the regulation of gene expression (Meister and Tuschl 2004), viral protection (Waterhouse et al. 2001; Wang et al. 2006) and genome maintenance (Lippman and Martienssen 2004). The biggest challenge in harnessing RNAi as a gene-silencing tool is introducing the dsRNA into individual cells to initiate the RNAi response. In some organisms this is less problematic because the cells are able to uptake dsRNA from their environment (Palauqui et al. 1997; Voinnet and Baulcombe 1997; Fire et al. 1998). Therefore, dsRNA only needs to be introduced into the body of the organism (through injection) (Fire et al. 1998) or provided to the organism environmentally (through feeding or soaking) (Tabara et al. 1998; Timmons and Fire 1998; Timmons et al. 2001). The dsRNA is then taken up by cells, and in some cases spreads from cell to cell. This process, which has been termed the systemic RNAi response, occurs in some

organisms including plants, planarians, the nematode *Caenorhabditis elegans* and the red flour beetle *Tribolium castaneum* (May and Plasterk 2005).

Systemic RNAi has only been studied thoroughly in plants and *C. elegans* (May and Plasterk 2005). The mechanism of dsRNA uptake and spreading is not conserved between plants and nematodes. In plants physiological structures such as the plasmodesmata and the phloem vasculature are responsible for the spreading of dsRNA species, while in *C. elegans* several essential genes have been identified in the dsRNA uptake process (May and Plasterk 2005). Therefore, the question of how other organisms that show a systemic RNAi response uptake dsRNA from their environment has yet to be answered.

In *C. elegans*, the transmembrane protein SID-1 has been identified as playing a major role in uptake of dsRNA from the external environment into somatic and germ-line tissues (Winston et al. 2002). While *sid-1* is critical for the RNAi response in *C. elegans* the conservation of this role in other animals has yet to be determined (Tomoyasu et al. 2008). *Drosophila melanogaster*, the other highly established invertebrate model organism, does not have a *sid-1* ortholog or a robust systemic RNAi response (Tomoyasu et al. 2008). Therefore, it has not been utilized as a genetic model for the cellular uptake of dsRNA.

Because *Drosophila* has neither a robust systemic RNAi response nor a *sid-1* ortholog it has been inferred that it is the presence or absence of sid-1 that determines whether an organism can or cannot respond to externally provided dsRNA (Winston et al. 2002; Dong and Friedrich 2005; Consortium 2006) (Duxbury et al. 2005). However, as more RNAi-based studies have been performed, it has been shown that this correlation does not always hold true. Mosquitoes do not possess a *sid-1* ortholog, but RNAi has been shown to be successful for multicellular stages in both *Anopheles gambiae* (Blandin et al. 2002) and *Aedes aegypti* (Zhu et al. 2003)

(Tomoyasu et al. 2008). In contrast, *sid-1*-like genes have been identified in the *Bombox mori* genome, but the application of systemic RNAi in this species has been largely unsuccessful (Tomoyasu et al. 2008). Additionally, the parasitic nematode *Haemonchus contortus* is affected by dsRNA when it is provided externally, by soaking, yet *sid-1*-like genes have not been found in its genome (Zawadzki et al. 2006) (Tomoyasu et al. 2008). Finally, while *Tribolium castaneum* does have 3 *sid-1-like* genes (*Tc-silA*, *silB*, and *silC*) and a robust systemic RNAi response, *Tribolium sil* genes appear to be more similar to *C. elegans tag-130*, a *sid-1*-related gene that is not required for systemic RNAi (Tomoyasu et al. 2008). Furthermore, functional data in *Tribolium* suggests that *Tc-sil* genes are not essential for dsRNA uptake in the flour beetle (Tomoyasu et al. 2008).

If the presence of *sid-1* is not essential for all organisms exhibiting a systemic RNAi response, then what other mechanism for dsRNA uptake exists? In addition to *sid-1* (also independently identified as *rsd-8*) four other genes (*rsd-2*, *rsd-3*, *rsd-6* and *sid-2*) have been identified as playing a role in dsRNA uptake in *C. elegans* (Tijsterman et al. 2004; Winston et al. 2007). Additionally, while most *Drosophila* cells do not have the ability to mount an RNAi response from externally provided dsRNA (Miller et al. 2008), it has been shown that *Drosophila* S2 cells do have the ability to actively take up long dsRNAs from their culture media (Clemens et al. 2000). To identify the genes essential for dsRNA uptake in *Drosophila* S2 cell culture two groups independently performed genome-wide RNAi screens and identified a number of genes as playing an essential role in dsRNA uptake (Saleh et al. 2006; Ulvila et al. 2006).

To determine whether any of these candidate genes play a role in dsRNA uptake in *Tribolium* cells we utilized an assay previously described by Tomoyasu *et al.* (2008) (also

described in the Material and Methods section). By this assay system we show that *Tc-epsin-like* (the *Tribolium* homolog of *C. elegans rsd-3*), the *Tribolium* homologs of *Drosophila CG5382* and *CG8671*, and the *Tribolium* homologs of several fucosylation pathway genes may play a role in the RNAi process in this beetle.

Material and Methods

Gene Cloning

Total RNA was extracted from three *Tribolium* pupae (Ga-1) using the RNeasy Protect Mini kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). *Tribolium* homologs of the candidate genes analyzed in this study were identified using reciprocal BLAST analysis of *Drosophila* or *C. elegans* homologs. PCR Primers were designed using the PrimerSelect module of Lasergene (DNASTAR, Inc.). PCR products were cloned into pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen).

dsRNA synthesis

dsRNA template was synthesized by PCR. For the large dsRNA fragments the entire cloned fragment was used as template (~500-600bp). A PCR primer corresponding to the sequence flanking the insertion site of the pCR4-TOPO vector with a T7 sequence at the 5' end was used in this PCR amplification (as previously described in (Tomoyasu et al. 2008)). For the smaller dsRNA fragments internal gene specific primers with T7 sequences at their 5' ends were utilized (~200bp). dsRNA was synthesized using the MEGAscript T7 High Yield Transcription kit (Ambion, Austin, TX, USA).

Injections

Larvae were injected as described previously (Tomoyasu and Denell 2004). In the *in vivo* assay system, dsRNA for the candidate gene (~40 larvae were injected with dsRNA for each candidate gene) was injected into Pu 11 larvae. Pu 11 is a transgenic line of beetles expressing EGFP in the wing primordia at the last larval stage and in the wings and eyes of the pupal stage. Two days after the injection of candidate gene dsRNA *EGFP* dsRNA was injected into a subset

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of the surviving larvae (a few larvae were left uninjected to act as a control). Three days later EGFP expression in the larvae and seven days later EGFP expression in the pupae was monitored. EGFP expression suggests that the candidate gene is involved in the RNAi process in *Tribolium* as the initial RNAi influenced the effectiveness of the *EGFP* RNAi. An absence of EGFP expression indicates that knockdown of the candidate gene had no effect on the ability of the organism to perform *EGFP* RNAi. This assay was previously used to illustrate the essential roles of *Tc-Dicer-2* (*Tc-Dcr-2*), *Tc-Argonaute-2a* (*Tc-Ago-2a*) and *Tc-Argonaute-2b* (*Tc-Ago-2b*) in the RNAi machinery in *Triboliu*m (Tomoyasu et al. 2008). With the publication of this assay system controls were also performed which illustrated that when dsRNA for genes not involved in the RNAi pathway (*dsRed* and *Tc-Ubx*) were knocked down via RNAi, EGFP was efficiently silenced (Tomoyasu et al. 2008). dsRNA for the candidate genes were injected at a concentration of 1ug/ul (~0.5ug/larva). dsRNA for *EGFP* was injected at a concentration of 0.01ug/ul (~0.005ug/larva) two days after the candidate gene injection. 30 minutes after the final injection larvae were placed on culture flour at 30C until documentation.

Documentation

Larvae were analyzed for EGFP expression 72 hours after the second injection. Pupae were analyzed for EGFP expression 1 week after the second injection. For documentation larvae were submersed in water. Water submersion immobilizes the larvae without causing lethality. After documentation larvae were dried briefly on a Kimwipe and returned to culture flour. Larvae and pupae were documented using an Olympus SZX12 microscope and a Nikon DXM 1200F digital camera. For each experiment identical exposure times were used for each image.

Results

We identified putative *Tribolium* homologs for systemic RNAi candidate genes found in either *C. elegans* (Table 1) or *Drosophila* S2 cells (Table 2). Of the candidate genes found in *C. elegans*, only *rsd-3* has an identifiable homolog in *Tribolium* (*Tc-epsin-like*) (Table 1) and other animals (Tijsterman et al. 2004). Of the 28 candidate genes found in *Drosophila* S2 cells, *Tribolium* has one to one putative orthology with all of them except for the SrC class of scavenger receptors (where *Drosophila* has four genes and *Tribolium* has only one) (Table 2). Orthologs of many of the genes identified in one of the two S2 cell screens were also found to be essential for the RNAi response in *C. elegans* (Saleh et al. 2006) (Table 2). To narrow the list of S2 cell candidate genes we cloned and analyzed only the 11 putative *Tribolium* orthologs identified as playing a critical role in RNAi in both *C. elegans* and S2 cell culture.

To test whether *Tc-epsin-like* (the homolog of *C. elegans rsd-3*) plays a role in dsRNA uptake in *Tribolium* cells we injected dsRNA for *Tc-epsin-like* followed by dsRNA for *EGFP* into Pu 11 larvae. EGFP expression was seen in the wing primorida in 41% of individuals (n=27) at the larval stage (Fig 1C). Unfortunately, RNAi of *Tc-epsin-like* appeared to cause lethality resulting in a low pupation rate, therefore EGFP expression at the pupal stage was not monitored. The illustration that RNAi of *Tc-epsin-like* did affect the knockdown of EGFP expression (Fig 1C) suggests that *Tc-epsin-like* plays an essential role in RNAi in *Tribolium*. To verify the specificity of the dsRNA used we performed RNAi of *Tc-epsin-like* using two smaller dsRNA fragments (Fig 1A). We found that regardless of the fragment of *Tc-epsin-like* dsRNA used EGFP expression was still seen after *EGFP* RNAi (Fig1C-E), indicating that *Tc-epsin-like* plays an essential role in the RNAi process in *Tribolium*.

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Each of the 11 genes identified as playing a role in dsRNA uptake in both *Drosophila* S2 cell culture and *C. elegans* was tested via the candidate gene assay. Eight out of the 11 genes showed a percentage of individuals expressing EGFP after *EGFP* dsRNA injection at the larval stage (*Arf72A*, *Gmer*, *CG5382*, *nina C*, *egghead*, *CG3911*, *CG8671*, *CG5161*) (Fig 2A). However, EGFP expression is still seen at the larval stage in 4% of EGFP control individuals (n=48) in which no candidate gene has been knocked down (Fig 2A). This is probably due to slight differences in the amount of *EGFP* dsRNA injected between individuals. Therefore, the expression seen at the larval stage in the experimental individuals may be due to incomplete knockdown of EGFP that is unrelated to the candidate gene knockdown. In EGFP control pupae (n=106) EGFP expression is never seen (Fig 2A, B), probably because more time has elapsed after the injection of *EGFP* dsRNA and therefore the knockdown is more efficient. Of the 11 genes analyzed only three still showed a percentage of individuals expressing EGFP at the pupal stage (*Gmer*; 15% (n=26), *CG5382*; 7% (n=27), *CG8671*; 5% (n=18)) (Fig 2A, D, F, J). Gmer is the fly ortholog of GDP-keto-6-deoxymannose 3,5-epimerase/4-reductase (GER) and plays an essential role in cellular fucosylation events (Roos et al. 2002; Rhomberg et al. 2006), CG5382 is a predicted zinc finger transcription factor with unknown targets and CG8671 has an unknown function (Table 2).

Because injection of dsRNA for these 3 candidate genes followed by injection of *EGFP* dsRNA resulted in EGFP expression at both the larval and pupal stage, they were considered the most likely to be involved in the systemic RNAi process in *Tribolium*. However, detailed functional information is only known for one, *Gmer*. Therefore, it was chosen for more extensive study. Gmer is an enzyme essential for the *de novo* synthesis of GDP-L-fucose (Roos et al. 2002; Rhomberg et al. 2006) (Fig 3A). GDP-L-fucose with the aid of fucosyltransferases

can be added to glycans or proteins through a process known as fucosylation. If fucosylation is important for the systemic RNAi response in *Tribolium*, as is expected by the EGFP expression seen in individuals in which Gmer function was knocked down, we would expect to see similar results by inhibiting other pathway members.

The fucosylation pathway begins when GDP-L-fucose is either synthesized from GDP-D-mannose or is obtained from the fucose salvage pathway. Once synthesized, fucose is transported to the Golgi apparatus via a GDP-L-fucose transporter. Fucosyltransferases in the Golgi then use GDP-L-fucose as a donor to fucosylate glycans and proteins (Roos et al. 2002) (Fig 3A). These modified sugars and proteins are known to be essential in a variety of cellular processes including inflammation, tumor metastasis, development and signal transduction (Roos et al. 2002). In *Drosophila* the two enzymes essential for the conversion of GDP-D-mannose to GDP-L-fucose are Gmd and Gmer (Roos et al. 2002; Rhomberg et al. 2006). The predicted GDP-L-fucose transporter is CG9620 and many fucosyltransferases have been identified (Roos et al. 2002).

When *Gmd* dsRNA followed by *EGFP* dsRNA was injected into Pu 11 larvae 10% of the resulting puape (n=30) showed some level of EGFP expression. When *CG9620* dsRNA followed by *EGFP* dsRNA was injected into Pu 11 larvae 21% of the resulting pupae (n=14) showed some level of EGFP expression. Removing the function of both Gmer and Gmd by RNAi increased the percentage of EGFP-expressing pupae from 15% and 10% respectively to 41% (n=8). As Gmer and Gmd are believed to act in the same pathway, this increase in EGFP positive individuals suggest that knockdown of Gmer and Gmd independently was not complete.

Discussion

The assay described in the above experiments was designed as an *in vivo* approach to establish whether candidate genes are essential for the RNAi response in *Tribolium*. However, because the Pu 11 line of beetles was the only line used for these studies this data only pertains to uptake of dsRNA into wing discs. The possibility remains that different tissues may utilize different methods of dsRNA uptake. Results from this assay must also be interrupted carefully as there is a possibility for both false positives and false negatives to be obtained. When multiple dsRNAs are present, competition can occur between these dsRNAs, which can influence the effectiveness of each dsRNA (McManus et al. 2002; Hutvagner et al. 2004; Bitko et al. 2005; Formstecher et al. 2006; Koller et al. 2006; Castanotto et al. 2007; Stierle et al. 2007). Such competition between the candidate gene dsRNA and *EGFP* dsRNA could result in incomplete knockdown of EGFP irrespective of the role of the candidate gene in the RNAi response, resulting in false positives. Controls for false positives, in which dsRNA for genes not involved in the RNAi response were injected followed by *EGFP* dsRNA, have been performed and published (Tomoyasu et al. 2008), but there could be differences in the competitive ability of each dsRNA (Formstecher et al. 2006; Koller et al. 2006; Yoo et al. 2008). False negatives are also possible in this assay system as anecdotal evidence suggests that the concentration of dsRNA required to knockdown gene function varies depending on the gene. If the candidate gene function was not efficiently knocked down by RNAi or if the candidate gene has a redundant role in the RNAi process, false negatives may be obtained. While we did not determine the level of gene knockdown after candidate gene dsRNA injection, dsRNA injection of five out of the 11 candidate genes resulted in individuals unable to eclose into adults (data not shown) and three out of the remaining six candidates that survived to sexual maturity seemed to

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have reduced fertility rates (data not shown). These results suggest at least some reduction in gene function.

Our results showed that for eight of the 11 candidate genes, individuals were expressing EGFP at the larval stage after *EGFP* RNAi and for three of the 11 candidate genes individuals were expressing EGFP at the pupal stage. However, the percentage of individuals expressing EGFP was low, varying from four to 25 percent. This low percentage is most easily explained by incomplete knockdown of the candidate genes, extended function of remaining proteins, or a functional redundancy of genes. However, it is important to note that even the knockdown of known RNAi mechanism genes does not result in 100% of individuals being insensitive to *EGFP* RNAi. We previously showed that only 58% of *Tc-Dcr-2* RNAi, 28% of *Tc-Ago-2a* RNAi and 42% of *Tc-Ago-2b* RNAi individuals showed EGFP expression after *EGFP* dsRNA injection (Tomoyasu et al. 2008). Inhibiting the function of RNAi component genes by RNAi may pose inherent difficulties, because the very genes being knocked down are required for the knockdown to occur. Therefore, it may not be possible to obtain long-lasting knockdown of RNAi components via RNAi.

While we focused on the candidate genes for which EGFP expression was seen in both the larval and pupal stages after *EGFP* RNAi, those for which EGFP was observed at the larval stage may still be involved. If the candidate gene knockdown was incomplete (due to insufficient dsRNA concentration or due to inherent problems with RNAi on genes essential for the RNAi process) then over time the small amount of remaining functional protein may be sufficient to allow cellular entry of enough *EGFP* dsRNA to result in EGFP knockdown. Even in the *Tc-Dcr-2* control there are fewer EGFP positive individuals at the pupal stage then at the larval stage, suggesting that over time the knockdown of Tc-Dcr-2 maybe less efficient.

While other candidate genes may play a role in systemic RNAi, the most likely candidates for involvement in the systemic RNAi pathway in *Tribolium* appear to be *Tc-epsin-like*, *Tc-Gmer* and the *Tribolium* homologs of *CG5382* and *CG8671*. The last three all yielded individuals expressing EGFP after *EGFP* RNAi at both the larval and pupal stages. *Tc-epsin-like* caused lethality before the pupal stage, but showed a much higher percentage of EGFP expression at the larval stage than did EGFP controls.

Gmer is known to play an essential role in the production of GDP-L-fucose, which can be added to proteins or other sugars through a process known as fucosylation (Roos et al. 2002; Rhomberg et al. 2006). Fucosylation has been shown to influence the binding of ligands to their receptors (Bruckner et al. 2000; Moloney et al. 2000). For example, in the Notch signaling pathway, activity of the glycosyltransferase Fringe increases the ability of the Notch receptor to bind its ligand Delta (Bruckner et al. 2000; Moloney et al. 2000). If there is a dsRNA receptor present on the surface of *Tribolium* cells, it is possible that fucosylation of the receptor may influence the ability of the receptor to bind dsRNA or other components necessary for the internalization of dsRNA. If this fucosylation pathway is essential for the uptake of dsRNA, the question remains why is the percentage of individuals showing a reduced systemic RNAi response so low? In vertebrates GDP-L-fucose can be synthesized via the *de novo* pathway from GDP-D-mannose, which requires both Gmer and Gmd or by the fucose salvage pathway, which is independent of Gmer and Gmd (Roos et al. 2002). Therefore, GDP-L-fucose may be provided via a mechanism independent of Gmer and Gmd making their presence not completely essential for fucosylation. The presence of a fucose salvage pathway offers a possible explanation for why the percentage of individuals showing a reduced systemic RNAi response in the Gmd and Gmer experiments is low, however, thus far there has not been a discovery of a fucose salvage

pathway in *Drosophila* (Roos et al. 2002). Therefore, a fucose salvage pathway may not exist in *Tribolium*.

Even if a salvage pathway is present in *Tribolium* and is responsible for the low percentage in the Gmer and Gmd experiments, the fucose transporter is required for fucosylation regardless of the mechanism of GDP-L-fucose synthesis and is therefore essential for fucosylation. The percentage of individuals showing EGFP expression after knockdown of CG9620 is higher than in either the Gmer or Gmd experiments however it still is not as high as the Dicer-2 control. CG9620 is thought to be the fucose transporter in *Drosophila* based on sequence similarity, however, this classification has not been functionally illustrated. Therefore, it is possible that CG9620 is not the fucose transporter or shares this role with other proteins. Alternatively, the percentage of individuals showing a reduced systemic RNAi response may be low because fucosylation may only act as a modulator of the dsRNA uptake process and may not be absolutely essential to the process.

The role that the other two S2 candidate genes (*CG5382* and *CG8671*) may play in the RNAi response in *Tribolium* is not as clear. CG5382 is a predicted zinc-finger transcription factor. As a transcription factor, this protein is unlikely to play a direct role in the uptake of dsRNA into *Tribolium* cells. However, it may play a role in regulating the transcription of genes essential for either the cellular uptake of dsRNA or the RNAi mechanism itself. CG8671 has an unknown function in *Drosophila* and therefore it is impossible to speculate on its mechanism of action in the RNAi response.

In addition to the genes identified from the S2 screens, one candidate gene was targeted due to its important role in dsRNA uptake in *C. elegans* germ cells. RSD-3 is not a transmembrane protein and thus its *Tribolium* homolog is unlikely to play a direct role in dsRNA

uptake. However, proteins with epsin N-terminal homology (ENTH) domains, like RSD-3, are known to play essential roles in clathrin-mediated endocytosis (Horvath et al. 2007). These proteins facilitate vesicle-mediated transport through the creation of membrane curvature and the recruitment of clathrin coat components to the membrane (Horvath et al. 2007). Therefore, it is possible that the inhibition of the RNAi response we see when Tc-epsin-like is knocked down is due to reduced internalization of dsRNA through clathrin-mediated endocytois.

The mRNA expression of *Tc-epsin-like* is ubiquitious during *Tribolium* embryogenesis (data not shown). Interestingly, *Drosophila* also contains an *rsd-3* homolog, *Dm-epsin-like*, whose mRNA expression pattern is limited to the salivary gland primordium, gut and muscle system during embryogenesis. If this gene is found to have a limited expression profile in other *Drosophila* lifestages, then its limited expression may contribute to the lack of a robust systemic RNAi response in *Drosophila*.

This work has utilized the candidate gene approach to identify genes involved in the systemic RNAi process in *Tribolium*. Candidate genes were taken from model organisms in which the process has been studied. The organism in which this process has been most thoroughly studied is *C. elegans*. *C. elegans* is distantly related to *Tribolium* and there is evidence that, even within nematodes, species may not uptake dsRNA in the same way (Felix 2008). Therefore, candidate genes from *C. elegans* may not be the most applicable for insect studies. *Drosophila* is much more closely related to *Tribolium*, but most *Drosophila* cells do not appear able to be influenced by external dsRNA (Miller et al. 2008) and therefore this process has not been studied thoroughly in *Drosophila*. While data does exist for dsRNA uptake in *Drosophila* cell culture, these cells are likely to be hemocytes which are very specialized cells and therefore the way they uptake dsRNA may not be indicative of how other insect cells uptake

dsRNA. In fact, we have recently shown that when dsRNA is injected into *Drosophila* larva, hemocytes are the only cells in which effective gene knockdown is seen (Miller et al. 2008).

To fully understand the genes involved in the systemic RNAi response there is a need for a forward genetic screen to be performed in more organisms that exhibit a robust systemic RNAi response. Unfortunately, the only method of dsRNA introduction currently available in *Tribolium* is through injection, which is a laborious process that is not ideal for high thoroughput screening. Further research into the susceptibility of *Tribolium* to environmentally provided dsRNA needs to be performed. Genetic screens in which *Tribolium* is mutagenized and then provided (either by injection or another proven method) with lethal dsRNA would allow genes essential for the RNAi process in *Tribolium* to be identified.

Using the candidate gene approach our data indicates that the *Tribolium* homolog of *C. elegans rsd-3* (*Tc-epsin-like*) plays an essential role in the systemic RNAi process. Our data also suggests that fucosylation may play an important role in dsRNA uptake in *Tribolium*. Further studies will reveal the degree to which the mechanism of systemic RNAi is conserved between organisms, which will not only aid in utilizing RNAi as a tool to study gene function in insects but may also provide information essential for the use of RNAi as a pest control tool.

Acknowledgements

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Table 1: Candidates based on systemic RNAi genes found in *C. elegans*

Ce Gene Name	Ce Gene ID	Ce Biological Function	Tc Gene Name	Tc Gene ID
sid-1	CO4F5.1	dsRNA uptake somatic cells	*sid1-like A (silA)*	Tc11760
			sid1-like B (silB)	Tc06161
			sid1-like C (silC)	Tc15033
sid-2	ZK520.2	dsRNA uptake gut		
rsd-2	F52G2.2	dsRNA uptake germ cells		
rsd-3	C34E11.1	dsRNA uptake germ cells	*epsin-like*	Tc12168
rsd-6	F16D3.2	dsRNA uptake germ cells		

Gene Name	Dm Gene ID	Dm Biological Function	Ce RNAi[✝]	Tc Gene ID
Arf72A	CG6025	Endosome transport	Yes	Tc08443
AP-50	CG7057	Endocytosis	No	Tc11923
Clathrin hc	CG9012	Endocytosis	Lethal	Tc15014
ldlCp	CG6177	Exocytosis	Yes/No	Tc10886
light	CG18028	Lysosomal transport	Yes	Tc15204
ninaC	CG5125	Rhodopsin mediated signaling	Yes	Tc14087
Rab7	CG5915	Endosome transport	Lethal/No	Tc06036
eater	CG6124	Inate immune response	Unknown	XP_969372*
Sr-CI	CG4099	Inate immune response		
Sr-CII	CG8856	Inate immune response	Unknown	Tc15640
Sr-CIII	CG31962	Inate immune response		
Sr-CIV	CG3212	Inate immune response		
Vha16	CG3161	ATP synthase/ATPase	Lethal	Tc11025
VhaSFD	CG17332	ATP synthase/ATPase	Lethal	Tc06281
Gmer	CG3495	Metabolism/Fucosylation	Yes	Tc14956
Pi3K59F	CG5373	Lipid metabolism	Yes/No	Tc00620
Saposin-r	CG12070	Lipid metabolism	Unknown	Tc00449
egghead	CG9659	Oogenesis	Yes/No	Tc08154
	CG4572	Peptidase	No	Tc02692
	CG5053	Signal transduction	No	Tc07768
	CG8184	Ubiquitin ligase	Unknown	Tc04152
	CG8773	Peptidase	No	Tc16254
	CG5382	Zinc finger transcription factor	Yes/No	Tc09067
	CG5434	Translation regulation	Lethal/No	Tc12172
	CG3248	Unknown	Unknown	Tc12410
	CG3911	Unknown	Yes	Tc14009
	CG8671	Unknown	Yes	Tc04825
	CG5161	Unknown	Yes	Tc07973

*XP_969372 is a NCBI prediction that partially mathces Tc_02053, however, Tc_02053 seems to be a chimera of at least three genes

[✝]Yes indicates that when RNAi for the homolog of this gene in *C. elegans* was performed the systemic RNAi response was effected so that subsequent RNAi using *Unc* dsRNA was not effective. No indicate that when RNAi for the homolog of this gene in *C. elegans* was performed the systemic RNAi response in not effected so that subsequent RNAi using *Unc* dsRNA was effective. Lethal indicates that RNAi for the homolog of this gene in C. elegans caused lethality so the test was not performed. Unknown indicates that the test was not performed. / indicates that two different results were obtained depending on the concentration of dsRNA used to remove the candidate gene function.

Figure 1

Functional analysis of the candidate gene from *C. elegans* **(A)** Illustration of the predicted *Tc-epsin-like* gene and the three regions the synthesized dsRNA corresponds with. **(B)** Pu 11 larvae injected with *EGFP* dsRNA. **(B insert)** Uninjected Pu 11 larvae. **(C-E)** Pu 11 larvae injected with *Tc-epsin-like* dsRNA followed by *EGFP* dsRNA. **(C insert)** Control larvae in which *EGFP* dsRNA was not injected following the *Tc-epsin-like* dsRNA.

A epsin-like predicted cds (1bp-1623bp)

epsin-like dsRNA (604bp-1256bp)

epsin-like dsRNA #2 (663bp-934bp)

epsin-like dsRNA #3 (1014bp-1255bp)

B Control

C epsin-like
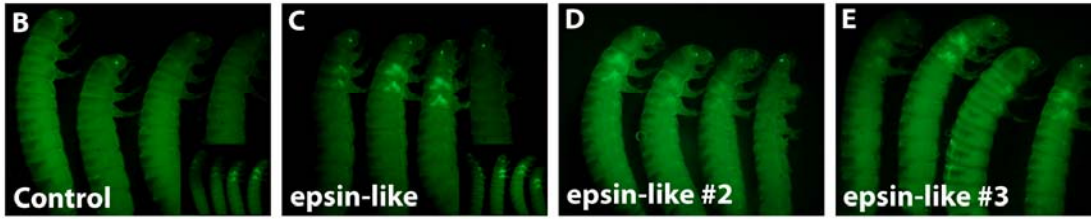
D epsin-like #2

E epsin-like #3

Figure 2

Functional analysis of the candidate genes from *Drosophila* S2 cells. **(A)** Bar graph illustrating the percentage of individuals at both the larval and pupal stages expressing EGFP after injection of candidate gene dsRNA followed by injection of *EGFP* dsRNA. **(B)** Pu 11 pupae injected with *EGFP* dsRNA. **(B insert)** Uninjected Pu 11 pupae. **(C-M)** Pu 11 pupae injected with candidate gene dsRNA followed by *EGFP* dsRNA. **(C-M inserts)** Control pupae in which *EGFP* dsRNA was not injected following the candidate gene dsRNA. Numbers on bar graph represent the number of positive individuals for EGFP expression/number of total individuals.
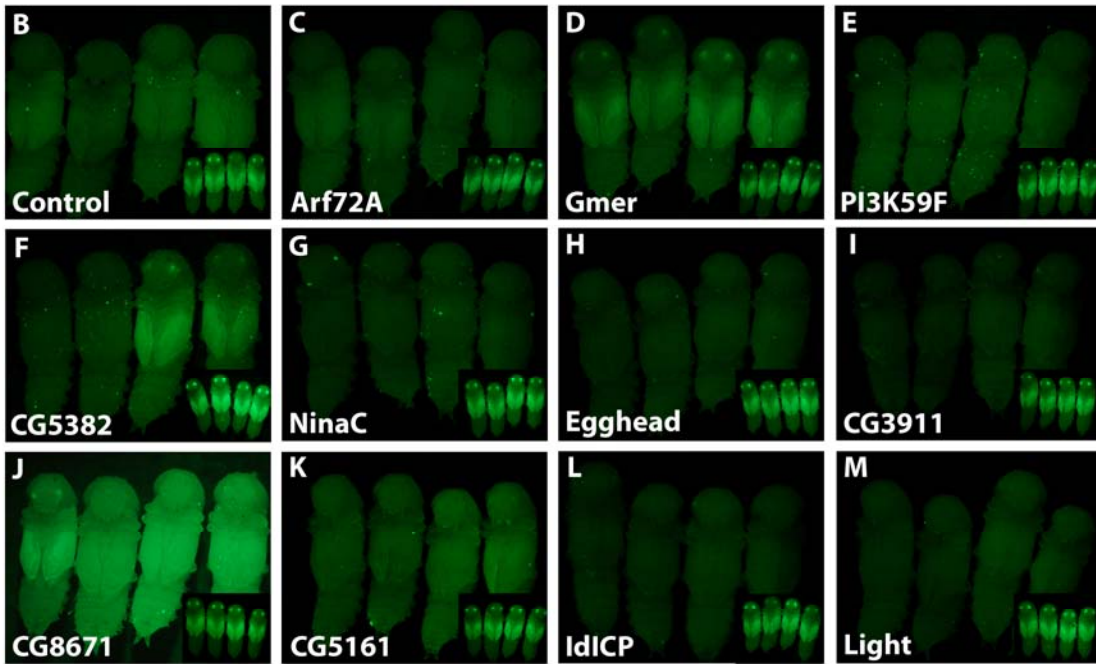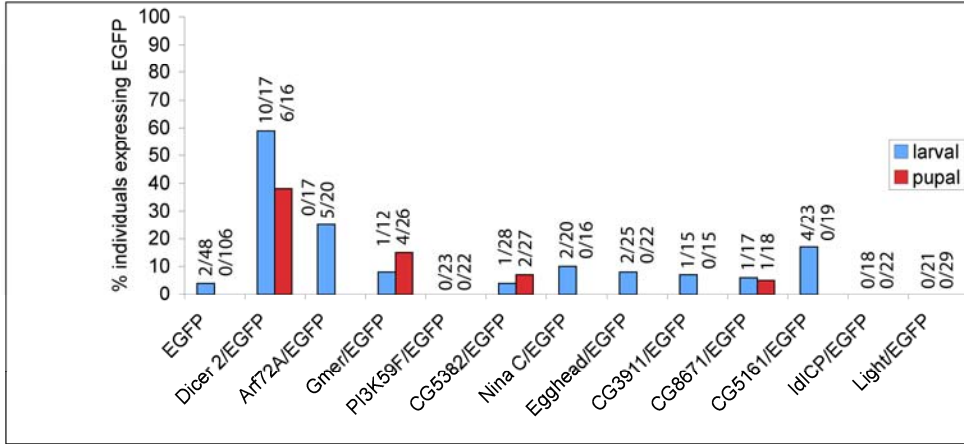
Figure 3

Functional analysis of genes involved in the fucosylation pathway. **(A)** Illustration of the fucosylation pathway. **(B)** Pu 11 pupae injected with *EGFP* dsRNA. **(B insert)** Uninjected Pu 11 pupae. **(C-E)** Pu 11 pupae injected with candidate gene dsRNA followed by *EGFP* dsRNA. **(C-E inserts)** Control pupae in which *EGFP* dsRNA was not injected following the candidate gene dsRNA.

A

GDP-D-mannose

GMD (Gmd)

GDP-4-keto-6-deoxy-mannose

GER (Gmer)

GDP-L-fucose

GDP-L-Fucose transporter (CG9620)

Golgi Apparatus

O-fucosyltransferases

Fucosylated glycan or protein

B Control

C Gmd

D CG9620

E Gmd & Gmer