THE ROLES OF TRANSIENT RECEPTOR POTENTIAL CHANNELS IN THERMOSTATIC BEHAVIOR, IN THERMAL ACCLIMATION, AND IN TONIC IMMOBILITY IN THE RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM* (COLEOPTERA: TENEBRIONIDAE)

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
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Abstract

Organisms are capable of sensing environmental conditions through diverse mechanisms. Transient receptor potential channels (TRPs) are a cation channel family that has been found to function in diverse sensing mechanisms. In this dissertation, I identified the function of several TRPs in thermosensing and mechanosensing in the red flour beetle, *Tribolium castaneum*. Candidate TRPs were chosen based on homology to TRPs found and studied in *Drosophila melanogaster*. To identify the function of candidate TRPs in *T. castaneum*, I suppressed the expression of target genes by RNA interference technique and investigated the phenotype of each treated beetle.

Temperature is a major limiting environmental factor for organisms. I tested the function of candidate TRPs in thermotaxis (behavior) and thermal acclimation (physiology). Using bioinformatics approaches, I identified three candidate TRPs – *painless*, *pyrexia*, and *trpA1* – involved in high temperature sensing. To test thermotactic behavior, I investigated beetle movement on a temperature arena with two separate temperature zones. Thermal acclimation was tested by pre-exposing beetles to either 42 °C for 10 min. When treated with double stranded RNA of TRPA1 (*dstrpA1*), the thermotactic response of beetles at 39 and 42 °C was reduced when compared to control groups. With pre-exposure at 42 °C, survivorship of *dstrpA1*-treated beetles significantly increased after one minute exposure at 52 °C compared to beetles that were not pre-exposed. With *dspainless* treatment, beetles showed lower response to thermal acclimation and lower long-term survivorship. Beetles treated with *dspyrexia* showed lower recovery after heat treatment without pre-exposure at 42 °C.

To identify the function of candidate TRPs in mechanosensing, I evaluated dsRNA treated beetles for survival, walking behavior, and tonic immobility. Treatment with *dsnompC* and *dstrpA5* resulted in failure in eclosion, causing 93 % mortality in both treatments. Survivors in *dsnompC* showed defects in elytra sclerotization. In *dsnanchung* and *dsinactive* treatments, adults showed abnormal walking behavior and reduced walking speed that were likely caused by defects of mechanosensing in folding of the joint between the femur and tibia. For tonic immobility, beetles with *dsnanchung*, *dsinactive*, *dswaterwitch* and *dsick2* (insect cytokine 2) treatments showed increased sensitivity to mechanical stimulation leading to tonic immobility.

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Chapter 1 - Introduction

Organisms are capable of sensing the environmental conditions through diverse mechanisms. Sensing mechanisms have been traditionally categorized into five types – sight, hearing, taste, smell, and touch. These categories and the associated terminologies are based on human sensing organs; eyes, ears, mouth, nose, and skin, respectively. Sensing mechanisms can also be categorized by the physical properties of the environmental stimuli: light, sound, molecules, and ionic strength of the medium. In addition to these traditional five senses, organisms have the ability to detect diverse stimuli including temperature, gravity, magnetic fields, barometric pressure, and possibly other physical properties.

Sensing occurs through cells or organs that convert these environmental stimuli to neural signals that are further processed in the nervous system. Integral to the cell membranes are numerous proteins with abilities to change membrane ion conductance, or to activate intra- cellular signaling cascades, thus generating neural activities in response to environment condition. Therefore, modern studies in sensory mechanisms include understanding the roles and the properties of specific membrane proteins responding to the stimuli. Among many other uses, identifying the sensory proteins and understanding their properties have application in pest control, such as development of insect attractant-based traps and repellents.

In my dissertation research, I focused on some possible roles of a group of integral membrane protein named transient receptor potential (TRP) channels that are

known to have diverse sensory roles, including thermosensing, mechanosensing, and mediating light sensing. I also tested the functions of insect cytokine (ICK), which was reported to have a hormonal function causing paralysis in a lepidopteran insect, as a possible downstream responder to extreme environmental stimuli.

One of major achievement in my research was development of assays to measure the activities of TRP channels using the outcome of the sensory functions that are relevant to survival in a changing environment. In addition, I developed assays to measure thermotactic behavior and thermal acclimation mediated through thermosensation, and assays of tonic immobility mediated through mechanosensation. By using the assays that I developed, the roles of TRP channels and ICKs in these biological processes were examined by using RNA interference (RNAi) techniques in a model organism, *Tribolium castaneum.*

In this chapter, I briefly review the biological importance and the mechanisms of thermosensing and mechanosensing and what is known about TRPs that is relevant to these mechanisms. Literature that provided the foundation for studies of thermosensing and mechanosensing and responses to thermal stress is reviewed. I also review what is known about TRP channels in a model insect *Drosophila melanogaster* that have been recently described for their role in various sensory functions.

Theromsensing

Temperature has profound effects on organisms at molecular, physiological, behavioral, and ecological levels (Zars, 2003). In addition, seasonality, relative humidity, length of day, and other environmental factors are closely linked to temperature in nature.

Also, maintaining the fundamental biochemistry of cellular metabolism is dependent on temperature. Therefore, proper responses to temperature are required for survival of organisms in fluctuating environments. Moreover, each organism has a specific temperature range within which it achieves optimum physiological, behavioral, and ecological functioning. Thus, animals have developed various physiological, behavioral, and ecological strategies to find and stay in favorable temperature conditions, and to overcome unfavorable temperature conditions. As endothermic animals, mammals have diverse strategies to regulate body temperature, such as controlling metabolic rate, sweating, shivering, and hibernation. However, ectothermic organisms, including insects, are more susceptible to temperature changes because of their limited abilities of thermoregulation. Moreover, insects are more vulnerable to temperature changes because of their small size, causing a large ratio of surface area to volume, and their small volume allowing less thermal tolerance. Under these biological constraints, the survival of insects requires proper short-term physiological and behavioral responses to temperature changes, long-term physiological changes, and evolutionary adaptation. Both behavioral responses and physiological changes are mediated by thermosensation and thermosensory-mediated responses (Zars, 2001).

Behavioral Response: Thermotaxis

Behavioral responses to unfavorable temperature conditions involve two complementary strategies: avoiding extreme thermal conditions that could be harmful or lethal (Grant and Dunham, 1998), and increasing the time spent at more physiologically optimal temperatures (Huey et al., 2003). Directed movement guided by temperature

differences is known as thermotaxis (Rosenzweig et al., 2005). This behavior is a rapid behavioral response to temperature changes. Therefore, it is easy and rapid to evaluate the temperature-sensing ability through thermotactic behavior. Thermotaxis helps organisms to escape from unfavorable or lethal temperature environment, or makes them stay more time in a favorable temperature environment. As a behavioral response, thermotaxis requires two separate abilities: to sense environmental temperature, and to execute the appropriate behavioral responses (Rosenzweig et al., 2005). This behavioral response has been described in diverse insects. The fruit fly, *D. melanogaster*, showed a strong behavior response on the thermal gradient plate with its distribution (Sayeed and Benzer, 1996). Two water scavenger beetles – *Tropisternus mixtus* and *Tropisternus columbianus* – were tested for their thermal preference behavior in a thermal gradient, but the strength of response was species dependent even though they are closely related species (Ybarrondo, 1995). The American cockroach, *Periplaneta americana*, also had thermal preference on the temperature gradient, and its antenna and tarsi were important for detecting the temperature differences (Murphy and Heath, 1983). Thermotaxis is a rapid response to temperature changes, so it is suitable to test the thermosensing ability and rapid behavioral responses on different temperature settings.

Physiological Response: Thermal Acclimation

Physiological changes in organisms caused by gradual temperature change conditions are known as thermal acclimation (Bowler, 2005; Loeschcke and Sorensen, 2005). These can involve changes in metabolic rate, enzyme activity, respiration rate, the nervous system, and the endocrine system (Neven, 2000). Thermal acclimation has been

described in various organisms. For example, in the western flower thrips, *Frankliniella occidentalis*, a short exposure to sub-lethal temperature increased the survival in the following exposure to a lethal temperature (Li et al., 2011), and in *D. melanogaster* temperature preference is affected in part by the thermal acclimation (Dillon et al., 2009). The proper acclimation responses require three steps: the detection of environmental cues, the transduction of detected information to a cellular response, and the activation of specific genes or proteins that increase thermal tolerance (Colinet et al., 2013).

A major mechanism for thermal acclimation is thought to be through induction of genes responsible for production of heat shock proteins (HSP); a well-known response to elevated temperature is the increased amount of HSPs within a short time period (Lindquist, 1986). The increased level of *hsp70*, the principle *hsp* in *D. melanogaster*, is involved in the thermo-tolerance enhancement (Welte et al., 1993; Feder et al., 1996; Gong and Golic, 2006; Bettencourt et al., 2008). With 20 minutes exposure to 40 °C, the increase of a heat shock protein and an *hsp* transcript was reported from the BCIRL-TcA-CLG1 (TcA) cell line (Goodman et al., 2012). Therefore, HSP can be accumulated with an acute heat stress in a short time period through gene regulation.

Thermal acclimation is not limited to high temperature. Seasonal cold acclimationis induced by temperature and photoperiod to enhance cold tolerance (Teets and Denlinger, 2013). However, various insects can rapidly enhance their cold tolerance within minutes to hours. This rapid physiological change is known as a rapid cold-hardening (RCH). In the flesh fly, *Sarcophaga crassipalpis*, the cold tolerance at -10 °C was significantly increased with 30 minutes pre-exposure at 0 °C (Lee et al., 1987). This low thermal acclimation is likely induced by rapid influx of calcium when the

environmental temperature was decreased. The calcium concentration of insect tissue was rapidly increased as the temperature was decreased (Teets et al., 2013).

Another category of thermal acclimation is through direct physiological responses without gene regulation. In the moss plant, *Physcomitrella patensi*, the influx of extracellular calcium ions through specific calcium-permeable channels in the plasma membrane during the beginning of temperature stress modulates the intensity of the heat shock response (Saidi et al., 2009).

The first step in thermal acclimation is detecting the proper cues. Therefore, identifying thermosensory mechanisms in *T. castaneum* is important to understand the initiation of thermal acclimation. In my dissertation study, thermotaxis and thermal acclimation have been focused as behavioral and physiological responses, respectively, to temperature changes.

Mechanosensing

Mechanosensing is the detection of changes in mechanical forces including touch, vibration, proprioception, balance, texture, and volume. This sensing mechanism responds to turgor in proportion to the surrounding concentration of water, so it is not specialized for specific stimuli (Kung, 2005). Mechanosensors, activated by mechanical stimuli, could be categorized into two groups: one that is directly activated by mechanical forces, and the other that is downstream of a messenger pathway from a non-channel sensor (Lin and Corey, 2005).

Tonic Immobility

Tonic immobility is a response to external stimuli believed to protect organisms against predators (Miyatake et al., 2004). It is also called thanatosis, apparent death, playing dead, and playing possum. This behavior has been reported from various organisms from insects to mammals (Francq, 1969; Sargeant and Eberhardt 1975; Gehlbach, 1970; Edmunds, 1972; Gibran, 2004). Tonic immobility is thought to be the consequence of the selection of prey behaviors by predation pressure, and this behavior is heritable (Prohammer and Wade 1981; Sih, 1992; Lima, 1998; Miyatake et al., 2004). When a prey perceives a predators, it has two alternatives tactics to escape from predation: immobilizing and fleeing. However, organisms cannot choose two tactics at the same time because these tactics conflict (Miyatake et al., 2008). Indeed, it has been reported that individuals with high level of tonic immobility showed less activity level in *Nasonia vitripennis* and *T. castaneum* (King and Leaich, 2006; Miyatake et al., 2008). Miyatake et al. (2008) showed a high level of dopamine in the brain reduced the duration of tonic immobility in a selected line of *T. castaneum*. Using selected lines, the long tonic immobility line was more sensitive to various stressors than the short tonic immobility line in *T. castaneum* (Kiyotake et al., 2014).

In this study, I manually induced tonic immobility as a response to a specific mechanical stimulus. With manually induced tonic immobility, I developed an assay to measure the mechanosensing ability of *T. castaneum*. It is easy to induce the tonic immobility and to measure the strength and duration of this behavior instantly. Therefore, this behavior is a good behavioral criterion to measure the mechanosensing ability of each *T. castaneum*.

Insect Cytokine

Cytokines are secreted signaling proteins that regulate various physiological and morphogenetic events including stress responses, immune responses, tissue remodeling, development, and hemolymph aggregation (Matsumoto et al., 2012). The first insect cytokine (ICK) that was isolated was a growth inhibitory factor from the hemolymph of an armyworm that was parasitized by a parasitoid wasp (Hayakawa, 1990; Hayakawa, 1991). The function of ICKs was identified as growth retardation (Hayakawa, 2006), paralysis induction (Skinner et al., 1993; Ha et al., 1999), cardioacceleration (Furuya et al., 1999), cell proliferation (Hayakawa and Ohnishi, 1998), immune cell stimulation (Tsuzuki et al., 2012), and stress response induction (Kiyotake et al., 2014). In *T. castaneum*, the expression levels of ICK between long and short tonic immobility lines were different when beetles were exposed to heat stress (Kiyotake et al., 2014). This observation led to investigate whether the ICK is involved in the tonic immobility as downstream commander of the sensory function. I tested the two ICKs among five ICK candidate genes identified in the *T. castaneum*.

Transient Receptor Potential Channels (TRPs)

Transient receptor potential channels (TRPs) are a family of six-transmembrane polypeptide subunits that make tetramers to form a non-selective permeable cation channel (Montell, 2005). Minke et al. (1975) described the first TRP channel in a white-eyed *trp* mutant strain of the fruit fly, *D. melanogaster*, that made transient rather than sustained responses to constant light, so the mutant fly showed defects in the visual response. TRPs are considered one of the ancient protein families because they are found

in all metazoan but not in land plants (Wheeler and Brownlee, 2008). TRPs are categorized into seven TRP subfamilies based on its homology (Clapham, 2003). Various kinds of TRPs are found in diverse organisms, and most of them are involved in sensory functions including vision, taste, smell, hearing, hygrosensation, and thermosensation (Mori, 1999; Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005; Liu et al., 2007; Rosenzweig et al., 2008). The function of TRPs is highly diverse with its specificity to different cations and its gating mechanisms (Venkatachalam and Montell, 2007). In addition, several TRPs are known as multimodal sensory function (Dhaka et al., 2006; Afroz et al., 2013).

I summarized the functions of the TRPs of the *D. melanogaster* in Table 1.1 along with stage- and tissue-specific expression pattern of the *trp* genes in Fig. 1.1 for which the raw data were obtained from FlyBase (http://www.flybase.org). In *D. melanogaster*, 13 TRPs have been described, while 27 TRPs have been identified in humans (Montell, 2011). Among seven subfamilies, TRPs in TRPA subfamily are sensors for high temperature (Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005). TRPL and TRP in TRPC subfamily have been described in cold temperature sensing (Rosenzweig et al., 2008). Nanchung in the TRPA subfamily and Waterwitch in TRPV subfamily are known for hygrosensing (Liu et al., 2007). TRPs in the TRPC subfamily are mainly involved in photoreception. Therefore, each subfamily of TRPs is specialized to sensing a specific modality.

In *D. melanogaster*, *pyrexia*, *nompC*, and *trpl* are highly expressed in the head parts including brain, head, and eye. These three TRPs are involved in thermoreception, mechanoreception, and photoreception, respectively. Mechanoreceptors *nompC* and

inactive were highly expressed in digestive organs; these mechanoreceptors are likely stretch receptors of digestive organ. A hygrosensor, *waterwitch,* is highly expressed in the crop, an organ for food storage. Sensing the water content in the food with this hygrosensor could be involved in maintaining homeostasis. Therefore, the known function of each gene is well matched with the functional requirements of the specific tissues in which they are expressed.

Thermo-TRPs

Some members of TRPV, TRPM, and TRPA subfamilies have been known as thermo-TRPs because these channels are activated in specific temperature ranges and involved in temperature information processing or protecting organisms from heat stress (Patapoutian et al., 2003; Lee et al., 2005). In mammals, six thermo-TRPs have been described. Four thermo-TRPs, TRPV1, TRPV2, TRPV3, and TRPV4, were activated by high temperature and TRPA1 and TRPM8 were activated by low temperature (McKemy, 2007). Five thermo-TRPs have been found in *D. melanogaster*, Painless, Pyrexia, TRPA1, TRP, and TRPL (Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005; Rosenzweig et al., 2008). Only one orthologous thermo-TRP was described from mammals and *D. melanogaster*. However, the specific temperature range for TRPA1 activation is different: low temperature for mammals and high temperature for *D. melanogaster* (Dhaka et al., 2007; Rosenzweig et al., 2005: Caspani and Heppenstall, 2009). Between rodent and primate, the cold sensitivity of each TRPA1 was different because of only one amino acid residue difference (Chen et al., 2013).

Montell (2011) summarized two activation modes of thermo-TRPs: the direct activation mode (Voets et al., 2004) and the indirect activation mode (Kwon et al., 2008). In the direct activation mode, cold- and heat- sensitive thermo-TRPs were activated by temperature changes and this change was detected by voltage-dependent activation curve with *trpM8* for cold and *trpV1* for heat in mammal (Voets et al., 2004). In the indirect activation mode, the responses to small temperature difference could be amplified through a signal pathway. For *trpA1* activation in *D. melanogaster* larvae, temperature sensor induced the change of G protein-coupled receptor (GPCR), and *trpA1* was activated after this GPCR activation as a downstream signaling pathway (Kwon et al., 2008). By this way, small temperature difference amplified the signal to activate the response. For noxious or harmful temperature sensing, thermo-TRPs with the direct activation have advantages to make fast response, but the indirect activation might be good to detect subtle temperature difference in the optimal temperature range (Kwon et al. 2008).

Thermo-TRPs in *D. melanogaster*

To analyze the phylogenetic relationship among TRPs in *D. melanogaster*, 13 TRP amino acid sequences were aligned by the CLUSTAL W program (Thompson et al., 1994). The phylogenetic relationships were inferred using the Minimum Evolution method with MEGA5 program (Kumar et al., 2008) (Fig. 1.1). Five TRPs out of 13 identified TRPs in *D. melanogaster* have been described as thermo-TRPs (Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005; Rosenzweig et al., 2008). These five thermo-TRPs belong to two TRP subfamilies, TRPA and TRPC. Three high temperatures

activated TPRs – *painless*, *pyrexia*, and *trpA1* – belong to TRPA subfamily and two low temperatures activated TRPs- *trp* and *trpL* – belong to the TRPC subfamily.

Tracey et al. (2003) described the first insect thermo-TRP*, painless*, associated with the behavioral response of *D. melanogaster* larvae to a probe heated to more than 38 °C. A stereotypical rolling behavior was described from wild-type larvae, and the time to respond to the heated probe was measured as a behavioral response. With the *Gal4/UAS* system and *painless* mutant, the comparison of this behavior between wild type and mutant type showed the function of *painless* in temperature-involved behavior. The mutant line took more time to respond to the heat (Tracey et al., 2003). With whole mount in situ hybridization, they found that *painless* was expressed in small number of cells in the nervous system. Therefore, *painless* was identified as the primary noxious heat detector in *D. melanogaster* because the temperature threshold for activation was consistent with that induces heat avoidance behavior (Sokabe and Tominaga, 2009).

Lee et al. (2005) described Pyrexia as another thermo-TRP in *D. melanogaster*. The gene structure was confirmed by sequencing cDNA and identified two alternate transcripts, *pyrexia*-PA and *pyrexia*-PB. They detected the activation of *pyrexia* above 40 °C with heterologous expression system, *Xenopus laevis* oocytes and HEK293T cells. To identify the behavioral response, they tested the special distribution of *pyrexia* mutant and wild type of *D. melanogaster* on the thermal gradient block. With immunostaining, they detected the expression of *pyrexia* in various peripheral nerves and the central nerves in embryos. In adults, *pyrexia* was expressed in sensory neurons lying beneath the bristles around eyes, neurons innervating the bristles on the back of thorax and neurons in maxillary palps, proboscis and antennae (Lee et al., 2005).

With RNA interference strategy to suppress *trpA1* expression, Rosenzweig et al. (2005) tested *trpA1* for its function as warm temperature sensing in *D. melanogaster* thermotaxis on the thermal gradient disk. They detected *trpA1* protein from a small number of central brain neuron, neuroendocrine cells of the corpus cardiacum, two pairs of cells adjacent to the mouth hook, and the developing gut. A small set of warm temperature activated anterior cell (AC) neurons with *trpA1* expression was important to temperature preference in *D. melanogaster* (Hamada et al., 2008). Four isoforms of *trpA1* were identified and one of the exon was critical to detect warm temperature in *D. melanogaster* (Zhong et al., 2012). In contrast, TRPA1 was activated in cold temperature ranges in mammals (Caspani and Heppenstall, 2009). Among mammals, only one amino acid difference between rodent and primate results in different sensitivity of TRPA1 to cold (Chen et al., 2013). Therefore, the difference of activation temperatures of TRPA1 between mammal and *D. melanogaster* can be explained with subtle changes of two TRPA1.

For cold temperature sensing, TRPL is essential for cold avoidance and also has significant effects on cold avoidance in behavioral test with various mutant lines (Rosenzweig et al., 2008). With various combinations of mutant lines, the molecular relationship between cold avoidance and hot avoidance was tested on the temperature gradient plate to show the specificity of each thermo-TRPs to be activated (Rosenzweig et al., 2008). The cold detecting TRP channels were located in the fly antenna, while hot and cold thermoreceptors in *D. melanogaster* were detected from distinct brain regions (Gallio et al., 2011).

TRPs as Mechanosensors in *D. melanogaster*

Sensing mechanical stimuli including touch, vibration, proprioception, balance, texture, volume and osmolality is known as mechanosensing. These mechanical stimuli result in change of turgor in specialized cells, mechanosensors. However, mechanosensing is different from other sensing mechanisms because it is not specialized for specific ligands (Kung, 2005). Mechanosensors, activated by mechanical stimuli, could be categorized two groups: one that is directly activated by mechanical forces, and the other that is downstream of a messenger pathway from a non-channel sensor (Lin and Corey, 2005). In *D. melanogaster*, Painless, Nanchung, Inactive, and NompC were identified as mechanoreceptors (Walker et al., 2000; Kim et al., 2003; Tracey et al., 2003). With *nompC* mutant line, the mechanosensory physiology of *D. melanogaster* was disturbed by virtually abolishing mechanosensory signaling in tactile bristles (Walker et al., 2000). The function of Painless was described as nociception (Tracey et al., 2003). Nanchung was identified as an essential component of the chordotonal mechanotransducer, and a role in proprioception in locomotion (Cheng et al., 2010). Nanchung and Inactive are required for mediating hearing in *D. melanogaster* (Kim et al., 2003; Gong et al., 2004). All of these candidate mechanosensors belong to TRP channels, specifically TRPA, TRPN and TRPV subfamilies.

TRPs in *T. castaneum*

In *T. castaneum*, 14 candidate TRPs were identified in a bioinformatics study (Matsuura et al., 2009). Based on sequence similarity with other TRPs, I added one more TRPs, *trpGamma* (TC007028, GenBank Accession number EFA02794.1), that belongs to

TRPC subfamily. Therefore, I have 15 candidate TRP sequences for *T. castaneum*. Based on *D. melanogaster* researches and phylogenetic study, I narrowed down to five candidate genes for potential thermo-TRPs in *T. castaneum*, *painless, pyrexia, trpA1, trpL*, and *trp*. Among five candidate TRPs, *painless, pyrexia*, and *trpA1* were targeted for high temperature sensing and thermal acclimation experiments. All of these three TRPs belong to TRPA subfamily. Based on previous researches, I expanded candidates for mechanosensor to eight TRP channels, five TRPs – *painless, pyrexia, trpA1, trpA5*, and *waterwitch* - belong to TRPA subfamily, one TRP – *nompC* - belongs to TRPN subfamily, and two TRPs – *nanchung*, and *inactive* - belong to TRPV subfamily. In addition, two ICKs were also tested for its function as mechanosensing mediator because they play key roles in a various physiological processes.

Research Objectives

The goal for this research is to investigate the function of candidate TRPs as thermosensors, and candidate TRPs and ICKs in mechanosensing, in *T. castaneum*. To achieve this goal, I measured thermotactic behavior and thermal acclimation to test candidate TRPs as thermosensors, and tested the function of TRPs and ICKs in mechanosensing with suppression of target gene expression by RNAi.

The detailed objectives as follow:

I. <u>To identify the functions of candidate TRPs in thermosensing in *T. castaneum*.</u>

With three candidate thermo-TRPs, I made a temperature arena comprised of two

different temperature zones to assess short-term thermostasis in the beetles. To assess long-term response, I analyzed the effects of thermal acclimation with heat treatment on the survivorship of beetle. With these experiments, I investigated the function of candidate thermo-TRPs in thermotaxis and the thermal acclimation in *T. castaneum* with different *dsRNA* injections to suppress target gene expression.

II. To investigate mechanosensing mechanism in *T. castaneum*. I investigated the mechanosensing mechanism in T. castaneum with eight candidate TRPs and two ICKs, . Survivorship and walking behavior was tested after treatment with dsRNA of candidate genes. In addition, the sensitivity to, and strength of tonic immobility induced by, a mechanical stimulus was examined to identify the function of target genes in mechanosensing mechanisms in *T. castaneum*.

Data obtained from these studies regarding the role of specific TRPs will serve as a foundation for further genetic, physiological, and ecological studies for thermosensing and mechanosensing, as well as opening possibilities for alternative pest control strategies for *T. castaneum* and related insects by disturbing thermosensing and mechanosensing mechanisms.

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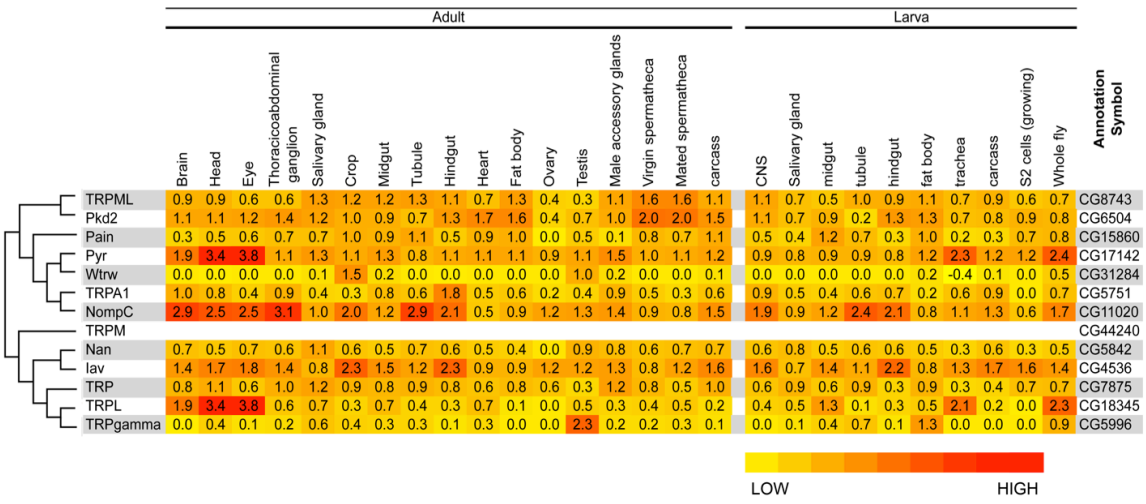

Fig. 1.1. Stage- and tissue-specific expression pattern of *Drosophila trp* genes.

The raw data were obtained from FlyBase (http://www.flybase.org). Relative expression levels were calculated based on the raw data with log transform. Each subfamily is well conserved in the phylogenetic analysis of 13 TRP amino acid sequences. The amino acid sequences were aligned by the CLUSTAL W program (Thompson et al., 1994). The phylogenetic relationships were inferred by minimum evolution method with MEGA 5 program (Kumar et al., 2008).

Table. 1.1. Summary for the *Drosophila trp* genes and its functions

Subfamily	Gene	Annotation symbol	Mode of activation	Tested with	Functions
TRPML	*trpml*	CG8743	N/A	N/A	N/A
TRPP	*pkd2*	CG6504	Ca2+ activated	Mutant line	Sperm flagella to sense directional cues, contractility of smooth muscle cells (Gao et al., 2004)
TRPA	*painless*	CG15860	>38'C Mechanical stimuli	Mutant line	Thermal and mechanical nociception (Tracey et al., 2003)
	pyrexia	CG17142	>40'C	Mutant line	Keeping thermal preference and increasing thermal tolerance (Lee et al., 2005)
	waterwitch	CG31284	Moist air	Mutant line and RNAi	Hygrosensing (Liu et al., 2007)
	trpA1	CG5751	>27'C	RNAi	Thermosensing and thermotaxis (Rosenzweig et al., 2005)
TRPN	*nompC*	CG11020	Tension	Mutant line Mutant line	Mechanosensory transduction (Walker et al., 2000; Cheng et al., 2010) Hearing (Eberl et al., 2000)
TRPM	*trpm*	CG44240		Mutant line	Intracelluar Zn(2+) homeostasis (Georgiev et al., 2010)
TRPV	*nanchung*	CG5842	Hypoosmolarity Dry air	Mutant line Mutant line and RNAi	Hearing (Kim et al., 2003; Gong et al., 2004) Hygrosensing (Liu et al., 2007)
	inactive	CG4536	Hypoosmolarity	Mutant line	Hearing (Gong et al., 2004)
TRPC	*trp*	CG7875	PLC dependent	Mutant line Mutant line	Phototransduction (Hardie and Minke, 1992) Cool avoidance (Rosenzweig et al., 2008)
	trpl	CG18345	PLC dependent	Mutant line Mutant line	Phototransduction (Niemeyer et al., 1996) Cool avoidance (Rosenzweig et al., 2008)
	trp gamma	CG5996	PLC dependent	Mutant line	Phototransduction (Xu et al., 2000)

Abbreviation: PLC, phospholipase C; RNAi, RNA interference

Chapter 2 - The Roles of Thermal Transient Receptor Potential Channels in Thermostatic Behavior and in Thermal Acclimation in the Red Flour Beetle, *Tribolium castaneum*

Abstract

To survive in variable or fluctuating environments, organisms require appropriate behavioral and physiological responses mediated through sensory mechanisms. When temperature is the environmental variable, organisms may respond with thermotaxis and/or thermal acclimation, behavioral and physiological responses, respectively, based on temperature sensing abilities. Transient receptor potential channels (TRPs) are a family of cation channels, a number of which, called thermo-TRPs, are known to function as thermosensors. I investigated the potential role of thermo-TPRs that have been previously identified in the fruit fly, *Drosophila melanogaster*, in thermotaxis and thermal acclimation in the red flour beetle, *Tribolium castaneum*. With phylogenetic analysis, the *trp* genes are generally conserved between *D. melanogaster* and *T. castaneum* as shown by one-to-one orthology, although I found putative gene-losses in two TRP subfamilies of *D. melanogaster*. With RNAi of other *D. melanogaster* TRP's found in *T. castaneum*, *painless*, *pyrexia* and *trpA1*, I measured thermal avoidance behavior in a behavioral assay. RNAi of *trpA1* resulted in reduced avoidance of high temperatures, 39 and 42 °C. I also measured the effects of RNAi on the heat-induced knockout and the death by a short exposure to high temperature, one minute at 52 °C, either with or without a 10-minute acclimation period at 42 °C. This acclimation treatment showed that relatively short time exposure to high temperature is enough to

induce high temperature thermal acclimation. RNAi of *trpA1* led to faster knockout at 52 °C. RNAi of *painless* showed lower recovery rates from heat-induced knockout after thermal acclimation, and RNAi of *pyrexia* showed lower long-term total survivorship without thermal acclimation. Therefore, I concluded that *trpA1* is important in high temperature sensing and also in enhanced tolerance to high-temperature induced knockout; *painless* plays a role in rapid acclimation to high temperature; and *pyrexia* functions in protecting beetles from acute heat stress without acclimation.

Introduction

Accurate temperature sensing is critical for organisms to survive in nature. Based on the accuracy of temperature sensing, organisms can stay in the temperature range that allows them to achieve their optimal functioning. Within specific temperature ranges, organism can maintain the fundamental biochemistry of cellular metabolism. Otherwise, organisms could encounter critical status. With relative small volume and large surface to volume ratio, insects are particularly susceptible to ambient temperature changes because of their limited abilities of thermoregulation as ectothermic animals (Gray, 2013).

Under these biological constraints, the survival of insects requires proper and rapid behavioral responses as well as long-term physiological acclimation to temperature changes, and both require the ability of the organism to accurately assess temperature. The relatively rapid behavioral responses involve two complementary strategies: negative thermotaxis avoiding extreme thermal conditions that could be harmful or lethal (Grant and Dunham, 1988), and positive thermotaxis increasing the time spent at more optimal temperatures (Huey et al., 2003). Thermal response requires two abilities: accurately

sensing environmental temperature and performing the appropriate behavior (Rosenzweig et al., 2005). As a long-term response, organism can acclimate itself physiologically to severe temperature with prior experience or gradual change of temperature (Bowler, 2005; Loeschcke and Sorensen, 2005). That is, with proper conditioning, organisms may enhance tolerance for severe or lethal temperature (Richter et al., 2010). Without thermal acclimation, accumulated heat stress can lead to death. Generally, it has been thought that physiological acclimation takes days (Sinclair and Roberts, 2005). However, rapid physiological change within one hour leading to low thermal acclimation also has been described (Lee et. al., 1987; Teets et al., 2013). The acclimation process requires in addition to accurate detection of environmental cues, the transduction of signals into a cellular response, and the activation of special genes, proteins and metabolites that increase thermal tolerance (Colinet et al., 2013).

In *Drosophila melanogaster*, behavioral response to temperature is affected by various factors including histamine and its receptors (Hong et al., 2006), rhodopsin (Shen et al., 2011) and transient receptor potential (TRP) channels (Tracey et al., 2003; Lee et al., 2005; McKemy, 2007; Rosenzweig et al., 2008). Among these various factors, TRPs have been found to play an important role as major temperature sensors that initiate thermal responses. TRP channels are six-transmembrane polypeptide subunits that make a tetramer to form a cation channel (Montell, 2005). TRPs are considered one of the most ancient protein families (Wheeler and Brownlee, 2008), and are involved in various sensing mechanisms including vision, taste, smell, hygrosensation, and thermosensation. (Mori, 1999; Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005; Rosenzweig et al., 2008; Montell, 2008). Among 13 TRPs in *D. melanogaster*, five thermo-TRP genes

have been identified; *painless*, *pyrexia*, and *trpA1* are involved in high temperature sensing, and *trp* and *trpL* in low temperature sensing (Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005; Rosenzweig et al., 2008).

TRPs have also been found in the beetle *Tribolium castaneum*, although their function has not been determined. In the present study, I identified the function of three candidate thermo-TRPs – *painless*, *pyrexia*, and *trpA1* - in *T. castaneum* for understanding detailed thermosensing at high temperatures. With this information, I could identify the role of each candidate thermosensor in different temperature induced behavior in *T. castaneum*, a model organisms as well as a stored-product pest. The phenotypes displayed by individuals after RNAi treatments suggested that these thermo-TRPs function for high temperature sensing and rapid acclimation to high temperature as they do in *Drosophila*.

Materials and Methods

Beetle cultures

I used *T. castaneum* strain GA-1 (Haliscak and Beeman, 1983) in this study. Beetles were cultured on whole-wheat flour containing 5 % of brewer's yeast by weight as a food source. Insects were reared at 30 °C with 16:8 (L:D) lighting condition and ca. 40% relative humidity (Beeman and Stuart, 1990).

Phylogenetic analysis of thermo-TRPs

The amino acid sequences of 13 *D. melanogaster* TRP channels, 14 *T. castaneum* TRP channels (Matsuura et al., 2009), and six human thermo-TRPs (McKemy, 2007)

were retrieved from NCBI. Further homology searches in NCBI databases, *Tribolium* genome sequences, expressed sequence tag database, and non-redundant *nr* database found one new *trp* gene that is orthologous to the *trpGamma* (TC007028, GenBank Accession number EFA02794.1), from the genome database of *T. castaneum*. The phylogenetic relationships were inferred for those total 34 sequences. The amino acid sequences were aligned by the CLUSTAL W program (Thompson et al., 1994). The phylogenetic analyses were made by MEGA5 program (Kumar et al., 2008) with minimum evolution method with 1,000 bootstrap resampling. The minimum evolution tree was built with the Close-Neighbor-Interchange (CNI) algorithm at a search level of zero. The evolutionary distances were calculated by the Poisson correction method, and were in the units of the number of amino acid substitutions per site (Fig. 2.1).

RNAi targeting thermo-TRPs and reverse transcription-quantitative PCR (RT-qPCR)

To synthesize DNA templates for double-stranded RNA (*dsRNA*), I targeted unique regions of each cDNA. The primers used for *dsRNA* synthesis and the nucleotide positions are shown in Table 2.1 and Fig. 2.2. The lengths of the regions for *dsRNA* syntheses for *dspainless*, *dspyrexia*, and *dstrpA1* were 707, 755, and 480 bp, respectively. The PCR products amplified with a pair of primers containing T7 promoter sequences at the 5' end were used for synthesizing *dsRNA*s by using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific). The *dsRNA* for *T. castaneum vermilion* was used as both a negative control for non-specific effects of *dsRNA* and as a positive control for monitoring effectiveness of RNAi by the loss of eye color. In addition, I used non-injected and buffer injected controls.

A total of 100 ng of *dsRNA* dissolved in 100 nl of injection buffer (0.1 mM sodium phosphate, 5 mM KCl, pH 7) with trace amount of food dye were injected into late stage of female pupae. I made six treatment groups, these include three controls: non-injected, buffer-injected, and *dsvermilion*-injected groups and three experimental groups: *dspainless*-, *dspyrexia*- and *dstrpA1*-injected groups. After treatments, all insects were kept in a growth chamber at 30 °C under standard conditions. Survivorship after injection was counted every other day to evaluate the mortality caused by injection wound. Within 10 to 14 days after emerging adults, *dsRNA* injected beetles were investigated for behavioral analysis, thermal acclimation, and heat-induced knockout.

Within one day after behavioral tests and heat acclimation tests were completed, total RNA was extracted from three to five female beetles from each treatment by Trizol reagent (Invitrogen) followed by Turbo DNase (Ambion) treatment to obtain DNA-free RNA based on the standard protocol. cDNA templates were synthesized from 500 ng of total RNA using ImProm-II reverse transcription system (Promega) with oligo (dT) 20-based protocol. Reverse transcription was followed by real-time PCR using SYBR premix Ex Taq (Takara Bio). Primers for the RT-qPCR assay were used for amplifying a 257 bp fragment for *painless*, a 532 bp fragment for *pyrexia*, a 176 bp fragment for *trpA1*, a 109 bp fragment for *vermilion*, and 182 bp amplicon encoding ribosomal protein S3 (*rps3*) served as a reference gene (Aikins et al., 2008) (Table. 2.1). The specificities of amplicons were accessed by melting curves analyses and electrophoresis on a 1.0% agarose gel. Mean Ct values for the each gene were calculated from two technical repeats and used to calculate $\Delta\Delta$Ct values (Livak and Schmittgen, 2001). Fold-

differences in the expression in each *dsRNA* injection treatment were calculated based on ΔΔCt values.

Behavioral analysis

To test thermotactic behavior, I designed a temperature arena assay with two temperature zones (Fig. 2.3). An oval shaped arena (1.5 x 5 cm) was separated two temperature zones by different temperature settings. The two zones were insulated by 2 mm of Teflon, and adjusted each for desired temperatures. Over the metal layer that controls the temperature, I placed a filter paper (Whatman No. 1) that had been kept with flour for more than a day; this was intended to create a somewhat "natural" setting for the beetles while still making it logistically possible to follow their movement. Oval-shaped cutouts in the 3 mm thick acrylic plate provided space for beetles on the filter paper layer. A layer of anti-glare glass covering the top was used to keep beetles inside of the arena. Beetles in the temperature arena were video recorded for 15 minutes from 30 second after the beetles were introduced. To analyze beetle behavior in the arena, time spent and speed in the different temperature zones were analyzed by EthoVision XT 7 (Noldus Information Technology, Wageningen, Netherlands). Based on EthoVision analysis, the percentage of time spent in the control zone (PTS) and the relative speed (RS) were calculated (Fig. 2.4). During the experiment, the temperature was monitored with four thermosensors with multi-channel thermometer, Center 304 Thermometer (Center Technology Corp., New Taipei City, Taiwan). The temperature was finely controlled within ± 1 °C range.

Five different temperature settings with six *dsRNA* treatments were tested. All experiments were performed in between 1 and 7 PM with a minimum brightness for video recording. I calculated averages and standard errors of each PTS and RS and compared each *dsRNA* treatment to buffer injected control with pairwise *t*-test ($p < 0.05$).

Thermal acclimation and heat-induced knockout

To evaluate the effects of the RNAi of different genes on thermal acclimation, I measured four factors in two separate experiments. With heat-induced knockout experiment, I measured the time to heat-induced knockout. Heat-induced knockout was defined as cessation of legs movement by exposure to high temperature at 52 °C in *T. castaneum*. With long-term acclimation experiment, I measured the maximum recovery rate from the knockout, the long-term survivorship, and the post-recovery lethality. I defined recovery as reestablishment of leg movement after heat-induced knockout; long-term survivorship was defined as survivorship at 20 days after heat treatment; and post-recovery lethality was defined as maximum recovery rate minus long-term survivorship.

To measure time to heat-induced knockout, each beetle was placed into a metal ring (2 cm diameter X 1 cm height) on a metal hot plate set as 52 °C. The time to heat-induced knockout was measured for six different *dsRNA* treatments – non-injected, buffer-injected, and *dsvermilion*-injected control groups and *dspainless*-, *dspyrexia*- and *dstrpA1*-injected experimental groups - either with or without acclimation for 10 min at 42 °C in a 0.2 ml polypropylene tube in a thermal cycler. After heat treatment, beetles were kept in 30 °C. Measurements were made within 30 minutes after the acclimation period. Three replications with ten individuals for each treatment were tested.

In long-term acclimation experiment, beetles were treated in a 0.2 ml polypropylene tube in a thermal cycler forcing them to be exposed either with or without acclimation for 10 min at 42 °C followed by 52 °C for 1 minute. One to two minutes after the 52 °C treatment, which include 1 minute in 30 °C in the thermal cycler and the time required for handling multiple individual at the same time, recovery rates and subsequent lethalities were recorded. Heat-treated beetles were kept in a petri dish with flour in a growth chamber at 30 °C under standard conditions. For each *dsRNA* treatment, ten females were tested per replication, and seven replications were made in total. Recovery and post-recovery lethality were assessed 0, 1, 3, 6,12, 24 hours and then every two days for 20 days after heat treatment. The maximum recovery rate and lethality were calculated and compared by average and standard error with pairwise *t*-test.

I did not find any difference between sexes with pairwise *t*-test ($p < 0.05$); therefore I only present data for females. I calculated averages and standard errors of each treatment and compared to control with pairwise *t*-test ($p < 0.05$) for each measurement.

Results

trp genes in T. castaneum

Phylogenetic analyses with the 34 TRPs of *Homo sapiens*, *D. melanogaster*, and *T. castaneum* suggested clear orthologous clusters of all seven subfamilies. While one-to-one orthology was generally conserved between *D. melanogaster* and *T. castaneum*, I found putative gene-losses in each TRPA and TRPC subfamily of *D. melanogaster*. Among five TRPA subfamily genes, three *T. castaneum* genes had an orthologous

relationship with more than 90 bootstrap scores to three thermo-TRPs described in *D. melanogaster*, *painless*, *pyrexia*, and *trpA1*, as well as other TRP gene, *Tcwtrw*. *TctrpA5* orthology in the TRPA subfamily was not found in *D. melanogaster* based on the phylogenetic analysis. Based on this phylogenetic analysis, I focused on the functions of three thermo-TRPs to identify in thermotaxis and in thermal acclimation. In the TRPC subfamily, which known for its functions in photoreception and cold temperature sensing (Rosenzweig et al., 2008), the *D. melanogaster* orthology of TcXP 970049 is missing. In addition, the phylogenetic relationship between *H. sapiens* and insects also showed the orthologous relationship.

The genes previously identified as thermosensors of high temperature in *D. melanogaster* were further analyzed for gene prediction and designing primers to generate *dsRNA* and to conduct RT-qPCR. I confirmed the structure of each candidate gene by cDNA sequencing; *painless* (TC007561, Accession number: EFA02836.1) is an intron-less gene with 2739bp open reading frame (ORF) on the linkage group 4, *pyrexia* (TC09731, Accession number: EFA07512.1) had three exons (213 bp, 176 bp, and 2275bp) covering the ORF on the linkage group 7, and *trpA1* (TC002449, Accession number: EFA01253.1) had 14 exons for 3939 bp the ORF on the linkage group 3.

Putative translation of these sequences in this study contained six transmembrane domains and six to 15 ankyrin repeat (Fig. 2.2). I also confirmed the mRNA structure and sequences of *painless* and *trpA1* by sequencing cDNAs covering full ORF.

RNA interference of thermosensing TRPs

Suppression of the target transcripts after *dsRNA* injection was confirmed by RT-qPCR and the eye color for *dsvermilion* injection. When compared to non-injected control, each *dsRNA* injection successfully suppressed the expression of target genes – *painless*, *pyrexia*, *trpA1* and *vermilion* (Fig. 2.S1). With RT-qPCR, I confirmed that *dsRNA* treatment suppressed the expression of target genes to 5 ~ 40 % of the expression of the non-injected control group. The mortalities of each *dsRNA* treatment within two days after injection, which was considered as lethality caused by injection wound, was less than 10 %. Therefore, I conclude that the mortality caused by RNAi injection of each *painless*, *pyrexia*, *trpA1* and *vermilion* was not significant.

Behavioral analysis

The percentage of time spent in the control zone (PTS) and the relative speed (RS) were analyzed to show the behavioral difference in different temperature zones and beetle's preference between two temperature zones (Fig. 2.4A). The sample size was summarized in Table. 2.2. For each temperature setting and *dsRNA* treatment, more than three biological replications were tested. As shown in Fig. 2.5, the PTS was not significantly different among three lower temperature comparisons, 30 vs. 30, 30 vs. 33, and 30 vs. 36 °C, except *dspyrexia* and non-injected control showed difference compared to the buffer injections in 30 vs. 30 and 30 vs. 36 °C regimes, respectively. However, the PTS was significantly higher, and differences among *dsRNA* injections were larger, at 30 vs. 39 and 30 vs. 42 °C. Among various *dsRNA* treatments, *dsRNA* injection of *trpA1* resulted in significant reductions in the PTS at high temperature settings compared to

buffer-injected control by pairwise *t*-test ($p < 0.05$), whereas the PTS of the others was dramatically increased at high temperature settings.

The relative speed was calculated by comparison between the speed in the experimental zone and that in the control zone (Fig. 2.6). The speed in the control zone (0.21 ± 0.07 cm/sec (Mean ± S.D)) was not significantly different among *dsRNA* treatments. At the three lower temperature settings (30, 33, and 36 °C), the differences among temperature settings and *dsRNA* treatments were not significant except for *dspyrexia* at 30 vs. 30 °C and *dstrpA* at 30 vs. 33 °C with relatively small differences. However, the relative speed was significantly increased at two higher temperatures (39 and 42 °C). *dstrpA1* injected individuals showed significant decrease in relative speed at 30 vs. 39 °C, but not at 30 vs. 42 °C, although a general pattern of reduced speeds was observed (Fig. 2.6). Instead, *dspainless* produced significantly reduced RS at 30 vs. 42 °C compared to the buffer-injected control.

Heat-induced knockout, recovery, and subsequent lethality

The time to knockout at 52 °C was not significantly different between male and female ($F = 1.68$; $df = 1$; $p = 0.1967$). The time required for heat-induced knockout with acclimation for 10 min at 42 °C was increased by two seconds compared to without acclimation treatment ($F = 25.64$; $df = 1$; $p < 0.001$). Among six different treatments, *dstrpA1* reduced the time to knockout compared to the control in both with- and without-acclimated group with pairwise *t*-test ($p < 0.05$) (Fig. 2.7). However, other treatments had no significant effect on time to heat-induced knockout in either with- or without-acclimated group.

Thermal acclimation significantly increased the recoveries from knockout in both controls and *dsRNA* treatments. Complete recovery occurred within 12 hours after the heat treatment in the acclimated beetles, while maximum recovery without acclimation reached in the days 2 to 4. Without acclimations, *dsvermilion* showed significantly higher recovery rate and reached to almost 100 % after 2 days. Another obvious difference was found in *dspyrexia* treatment having significantly lower in the maximum recovery (57 %) in the beetles without acclimation.

Significant levels of post-recovery lethality, 2 to 40 %, were observed up to 20 days of observations. Interestingly, *dsvermilion*-treated beetles, without acclimation, had significantly lower post-recovery lethality compared to the control treatment. The beetles treated with *dspainless*, with acclimation, had significantly higher lethality than buffer injected control. As a consequence, the long-term total survivorships in acclimations at the 20th day (Fig. 2.8F) were higher than those without acclimation in all *dsRNA* treatments. The *dspainless*, with acclimation, had significantly lower survivorship than buffer injected control. Without acclimation, *dsvermilion* had higher survivorship, whereas *dspyrexia* showed significantly lower survivorship compared to the buffer injected controls.

Discussion

TRP channels are categorized into seven TRP subfamilies by their homology (Clapham, 2003). A number of members of the TRPV, TRPM, and TRPA subfamilies are known as thermo-TRPs for the gating properties of the channels at different temperatures (Patapoutian et al., 2003). In mammals, six thermo-TRPs have been

described; four thermo-TRPs, *trpV4*, *trpV3*, *trpV1* and *trpV2*, were activated by high temperature and two, *trpA1* and *trpM8*, were activated by low temperature (McKemy, 2007). Five thermo-TRPs have been found in *D. melanogaster*; *painless*, *pyrexia*, *trpA1*, *trp*, and *trpL* (Tracey et al., 2003; Lee et al., 2005; Rosenzweig et al., 2005; Rosenzweig et al., 2008). Interestingly, the orthology relationships determined by the sequences are not parallel with the functions between the TRPs of *D. melanogaster* and mammals. Only one common thermo-TRP in mammals and *D. melanogaster*, *trpA1*, has been described for the thermosensing function (McKemy, 2007).

The most recent common ancestor of *T. castaneum* (Coleoptera: Tenebrionidae) and *D. melanogaster* (Diptera: Drosophilidae) existed approximately 300 mya (Grimaldi and Engel, 2005). Despite the time since their divergence, I found that the orthologous functions of *trpA1* as a channel sensing high temperature were conserved in these two species. However, the specific temperature range for *trpA1* activation is different in mammals and *D. melanogaster;* low temperature for the former and high temperature for the latter (Dhaka et al., 2007; Rosenzweig et al., 2005; Caspani and Heppenstall, 2009). This observation showing unexpected divergent functions of TRPs suggests that the thermosensing mechanisms are not evolutionarily stable ancestral characters.

Comparing my findings to studies done on these thermo-TRPs in *D. melanogaster*, I found a noticeable difference in the range of temperature that induced behavioral responses. In *T. castaneum*, *trpA1* affected behavior at 39 and 42 °C, but not at lower temperatures, whereas in *D. melanogaster trpA1* was the thermosensing channel for 24-29 °C (Viswanath et al., 2003). The other thermo-TRP channel candidates, *painless* and *pyrexia*, which were previously described in *D. melanogaster* for

thermosensing sensitive to 38 and 40 °C, respectively (Tracey et al., 2003; Lee et al., 2005), showed no effect on behavior of *T. castaneum* up to 42 °C, while they are involved in the tolerance to high temperature in this study. Therefore, the property of the *trpA1* in *T. castaneum* activated by the higher temperature than the temperature for activation of *trpA1* in *D. melanogaster* may be the consequence of evolution for their optimal temperature of the habitat.

High temperature sensing through *trpA1* is related to resistance to high-temperature induced knockout in *T. castaneum* without any effect on long-term survivorship after heat treatment (Fig. 2.8F). Other thermo-TRPs, *painless* and *pyrexia*, is likely protecting beetle from heat stress at lethal temperature range. However, *trpA1* is likely detecting the subtle temperature different below lethal temperature. Without acclimation, *pyrexia* RNAi resulted in a significant lower maximum recovery rate after heat treatment (Fig. 2.8D). In *D. melanogaster*, *pyrexia* protects insects from high-temperature stress and makes flies to keep the temperature preference (Lee et al., 2005). The individuals failed to recover from the knockdown eventually died in the subsequent counting. Therefore, I speculate that *pyrexia* is required for protecting beetles from sudden heat stress or waking up from the knockdown that is a critical in surviving from the heat stress. RNAi treatment of *painless* had lower ability for thermal acclimation, which is shown by lower long-term survivorship after 10-minute pre-exposure to high temperature (Fig. 2.8E and F). Since *painless* RNAi without thermal treatments showed no significant reduction of survivorship at the equivalent age (data not shown), the lower long-term survivorship in acute heat after acclimation is likely caused by suppression of *painless* expression. However, *painless* RNAi without acclimation did not show any

different patterns in the time to heat-induced knockout, the maximum recovery rate, and the long-term total survivorship compared to the buffer injected or non-injected control.

A 10-minute pre-exposure to high temperature was sufficient to acclimate *T. castaneum* to a subsequent acute high temperature. This type of acclimation, unlike long-term acclimation that involves gene regulation, is likely mediated by sensory mechanisms, as it was also shown to be the case with *pyrexia* in *D. melanogaster* protecting flies from paralysis within three minute of exposure to high temperature (Lee et al., 2005). For rapid thermal acclimation, the intracellular elevation of calcium ion was strongly induced by cold temperature (Teets et al., 2013). As a cation channel, TRPs can contribute to this calcium influx to induce thermal acclimation by sensing temperature change. The results in *T. castaneum*, indicating the protective functions of *painless* and *pyrexia* with acclimation and without acclimation, respectively, support the conclusion that *pyrexia* and *painless* are involved in both sensing of and protection from high temperature in a short time. By sensing the lethal temperature, it can induce the protecting mechanism against heat stress.

On the other hand, Goodman et al. (2012) showed that the expression of a heat shock protein (*hsp68a*) was significantly induced by only a 20-minute exposure to 40 °C heat treatment in an established cell line from *T. castaneum*. Similarly, a 10-minute exposure to 42 °C was enough for a significantly increased expression of *hsp68a* in the cell line (Park, personal communication), which likely also occurs at the whole organismal level. Based on these observations, a 10-minute exposure to 42 °C is enough to trigger a genomic level response for protecting organism from acute heat stress. Together with the results in this study which have shown the involvement of TRPs in the

rapid acclimation at the physiological levels, I conclude that both gene regulation and physiological response are likely responsible for the rapid acclimation to high temperature. Further study is needed to understand whether and how the induction of heat shock protein expression and the TRP mediated physiological changes are interacting in the rapid thermal acclimation.

Unexpectedly, I found that *vermilion*, which was originally included as a control, positively affected levels of resistance to thermal stress in beetles that had not been acclimated, in terms of both recovery rate and long-term survivorship (Fig. 2.8 E and F). The function of *vermilion* is known to encode tryptophan oxygenase (Lorenzen et al., 2002), which converts tryptophan to N-formyl-kynurenine. Previous reports have shown that mutation of the *vermilion* gene in *D. melanogaster* resulted in longer life span (Oxenkrug, 2010). I speculate that impaired formation of kynurenine, which can be cytotoxic metabolic product, is the cause of the phenotype. It is possible that *vermilion* RNAi blocked kynurenine production and may be linked to resistance to acute thermal stress, although the molecular mechanism requires further investigations.

Thermosensing is the key component to initiate the behavioral and physiological responses to maintain the organismal homeostasis and the fundamental biochemistry of cellular metabolism. In this study, I showed that *T. castaneum* can generate proper behavior against temperature change, protect itself from sudden heat stress, and rapidly acclimate itself to lethal temperature. All of these physiological and behavioral responses are based on proper thermosensing. RNAi mediated suppression of thermo-TRP candidates in *T. castaneum*, *painless*, *pyrexia* and *trpA1*, demonstrated that the candidate TRPs are involved in sensing high temperature and rapid acclimation to high temperature.

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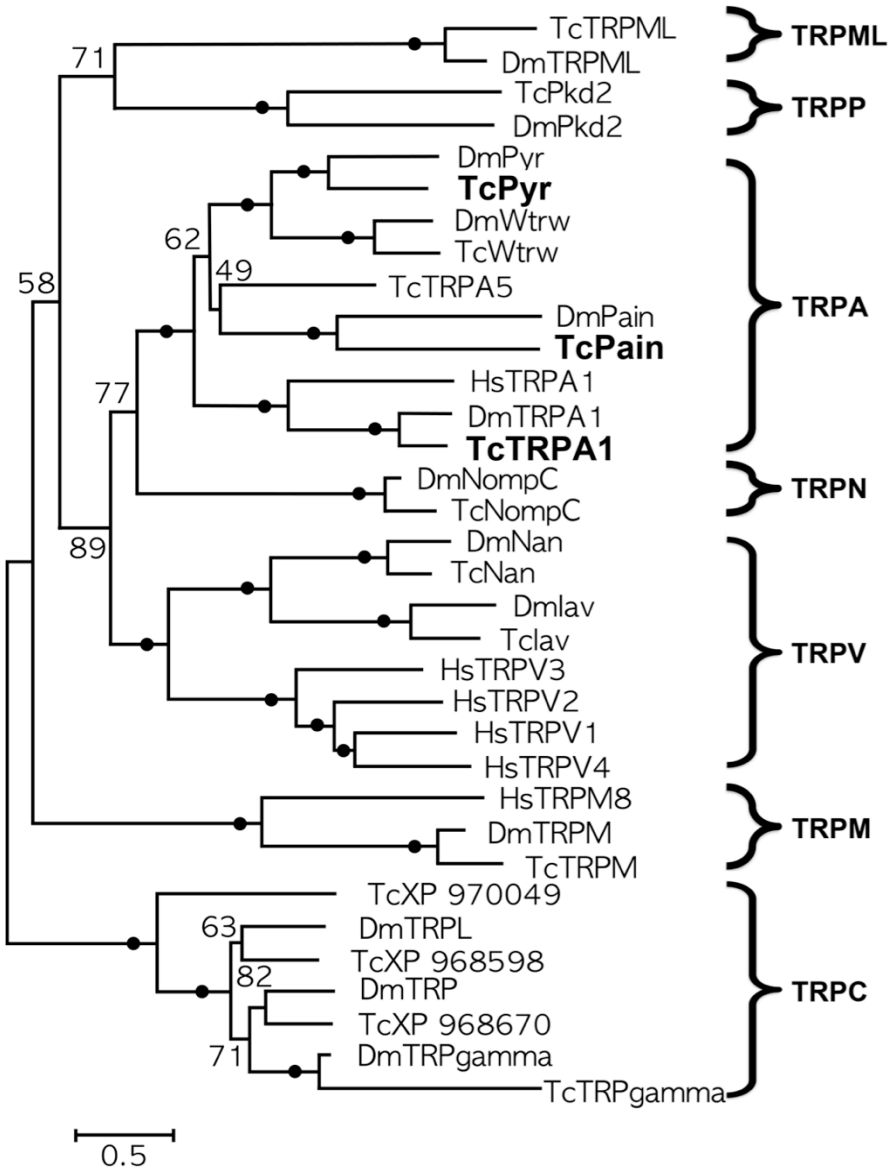

Fig. 2.1. Phylogenetic tree for 34 amino acids of TRPs.

The amino acid sequences of 13 *D. melanogaster* TRPs, 14 *T. castaneum* TRPs, and six human thermo-TRPs were aligned by the CLUSTAL W program. The phylogenetic tree was made by MEGA5 program with minimum evolution method with 1,000 bootstrap resampling. The solid circle indicates the bootstrap score was more than 90.

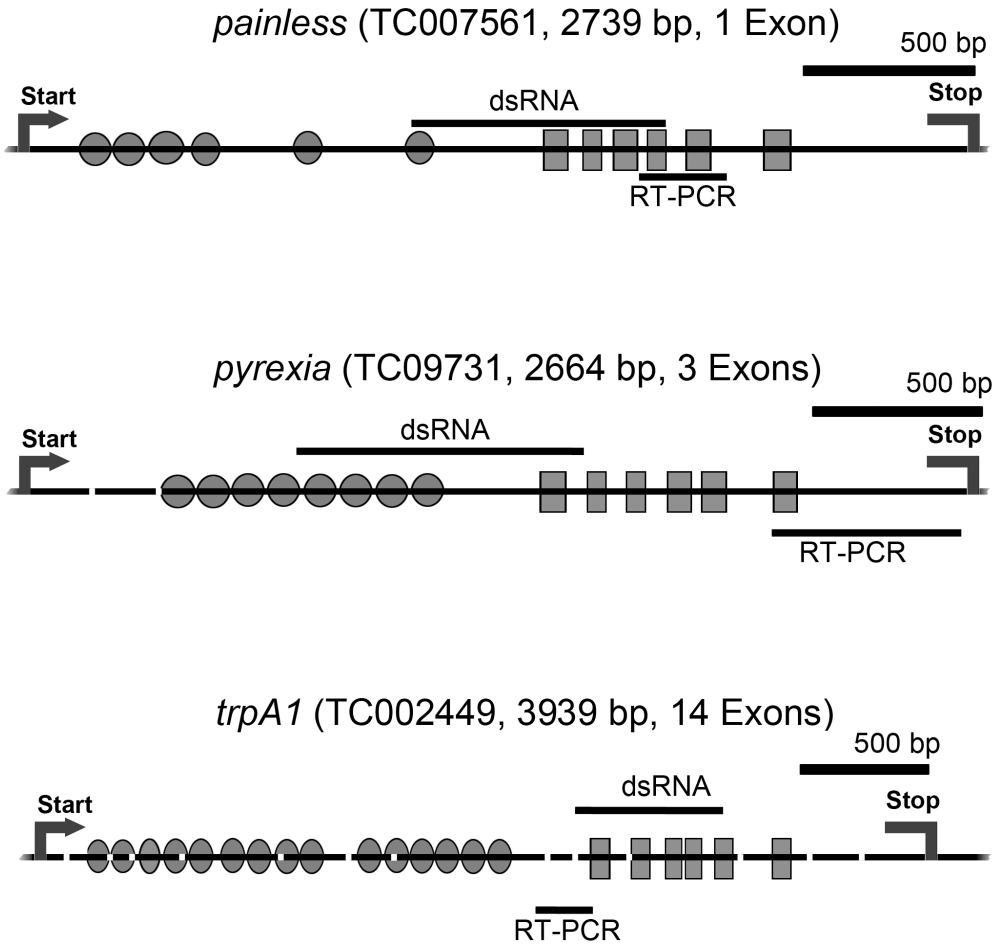

Fig. 2.2. Open reading frames (ORF) of three candidate genes.

The round shape represents the ankyrin motif and the rectangular shape is transmembrane domain. The targeted *dsRNA* region and qRT-PCR regions are noted.

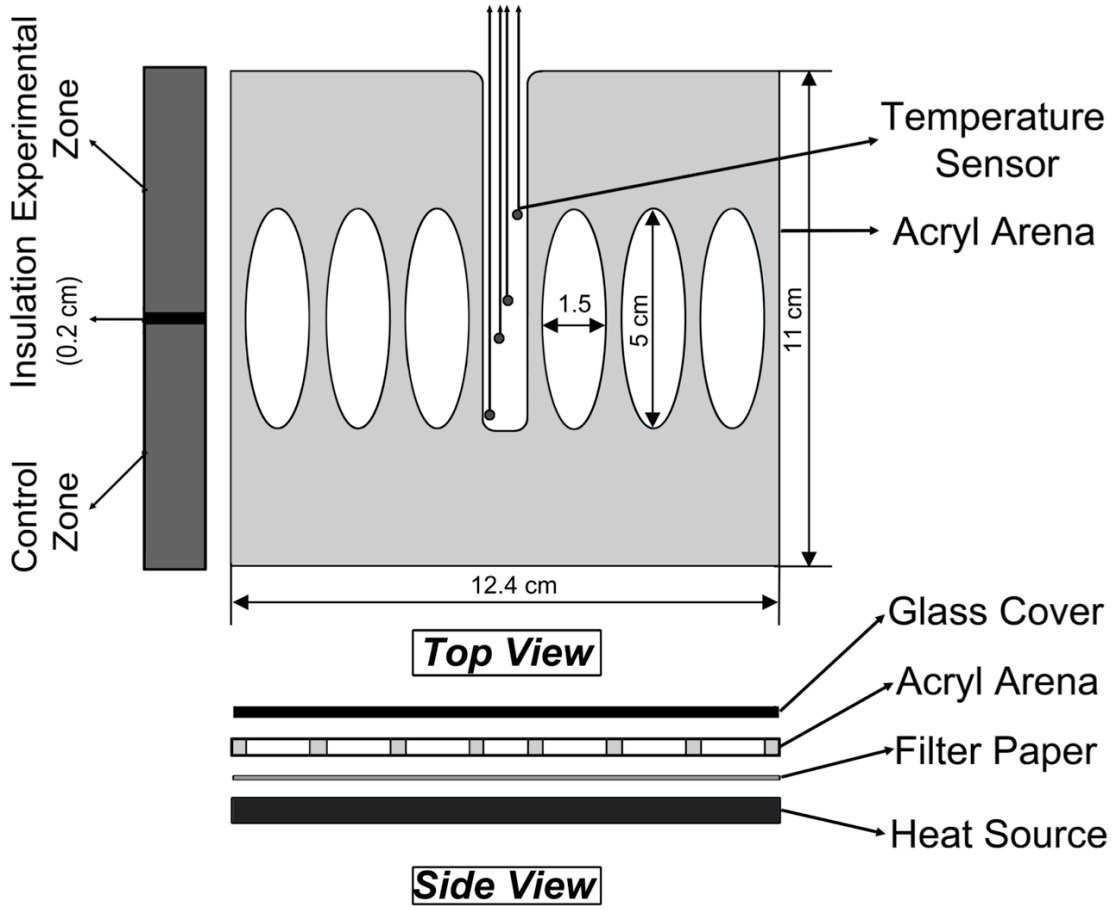

Fig. 2.3. Temperature arena.

See the description in the text.

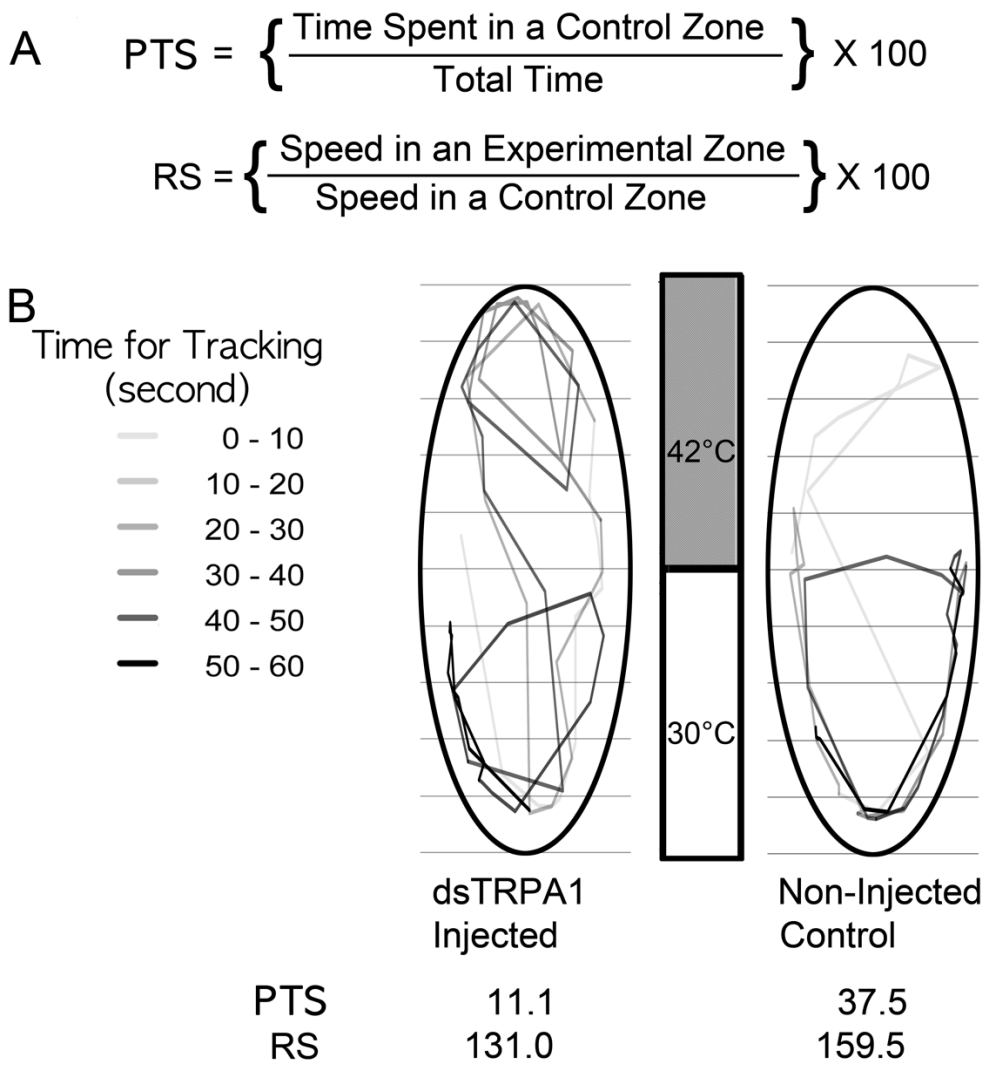

Fig. 2.4. Behavior analysis.

A. Calculation of the percentage of time spent (PTS) and relative speed (RS). B. An exemplary analysis of a beetle's movement for 1 min. in the temperature arena at 30 vs. 42 °C setting.

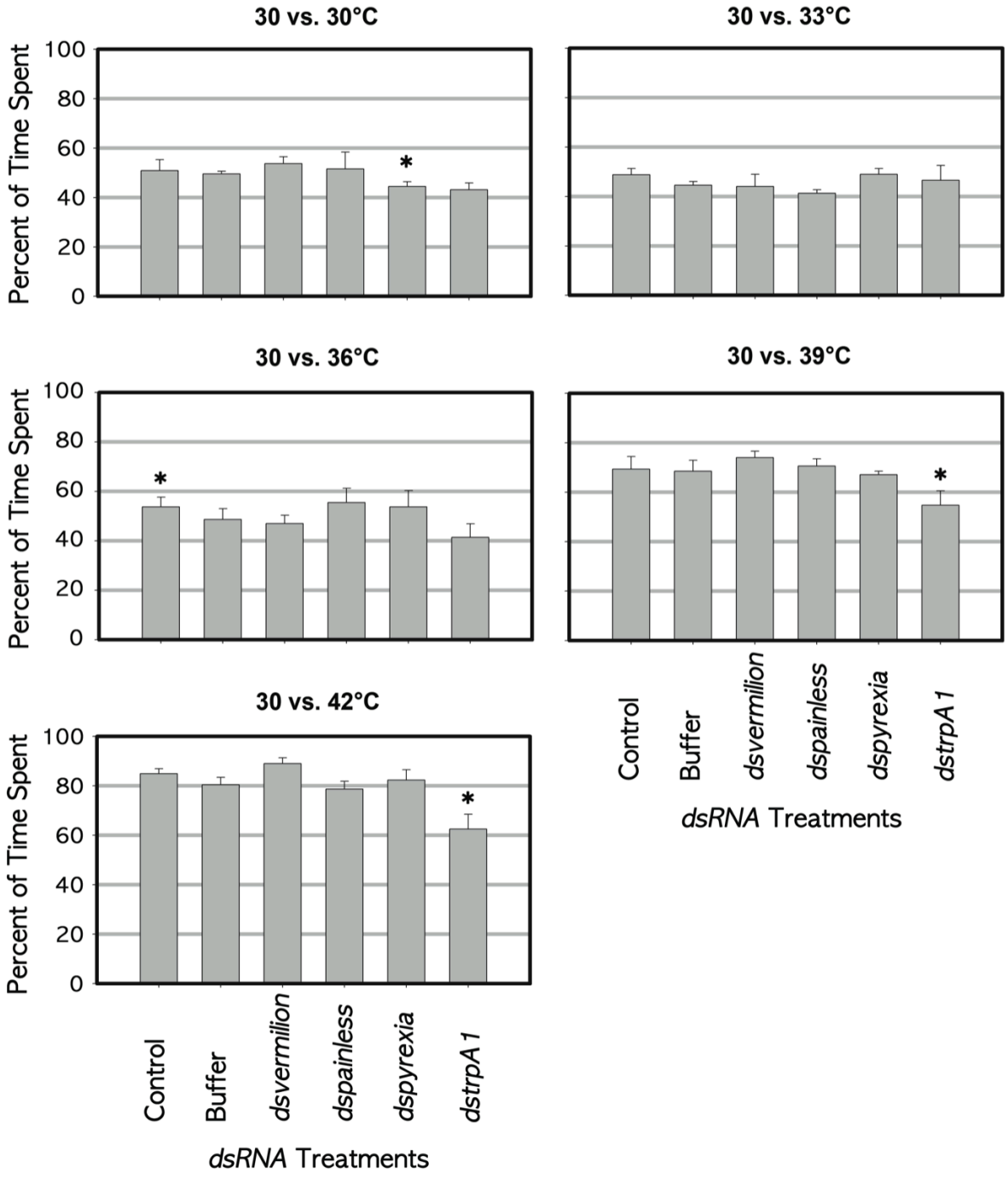

Fig. 2.5. Percentage of time spent (mean and standard error) of six *dsRNA* treatments in five temperature comparisons.

Within each temperature comparison, each *dsRNA* treatment was compared to a buffer-injected control by pairwise *t*-test. * indicates $p < 0.05$. The sample size is presented in Table 2.2.

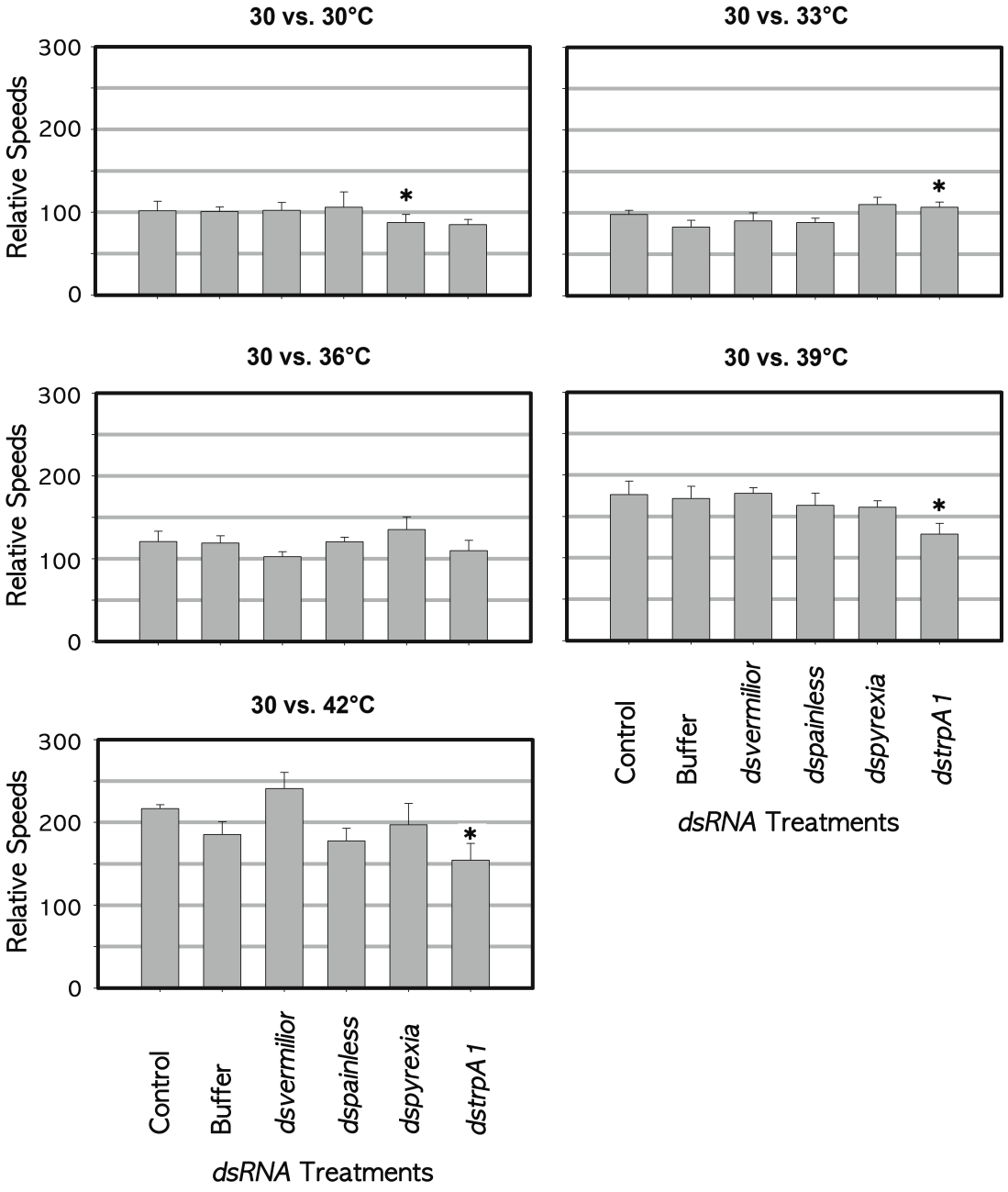

Fig. 2.6. Relative speed (mean and standard error) of six *dsRNA* treatments in five temperature comparisons.

Within each temperature comparison, each *dsRNA* treatment was compared to a buffer-injected control by pairwise *t*-test. * indicates $p < 0.05$. The sample size is presented in Table 2.2.

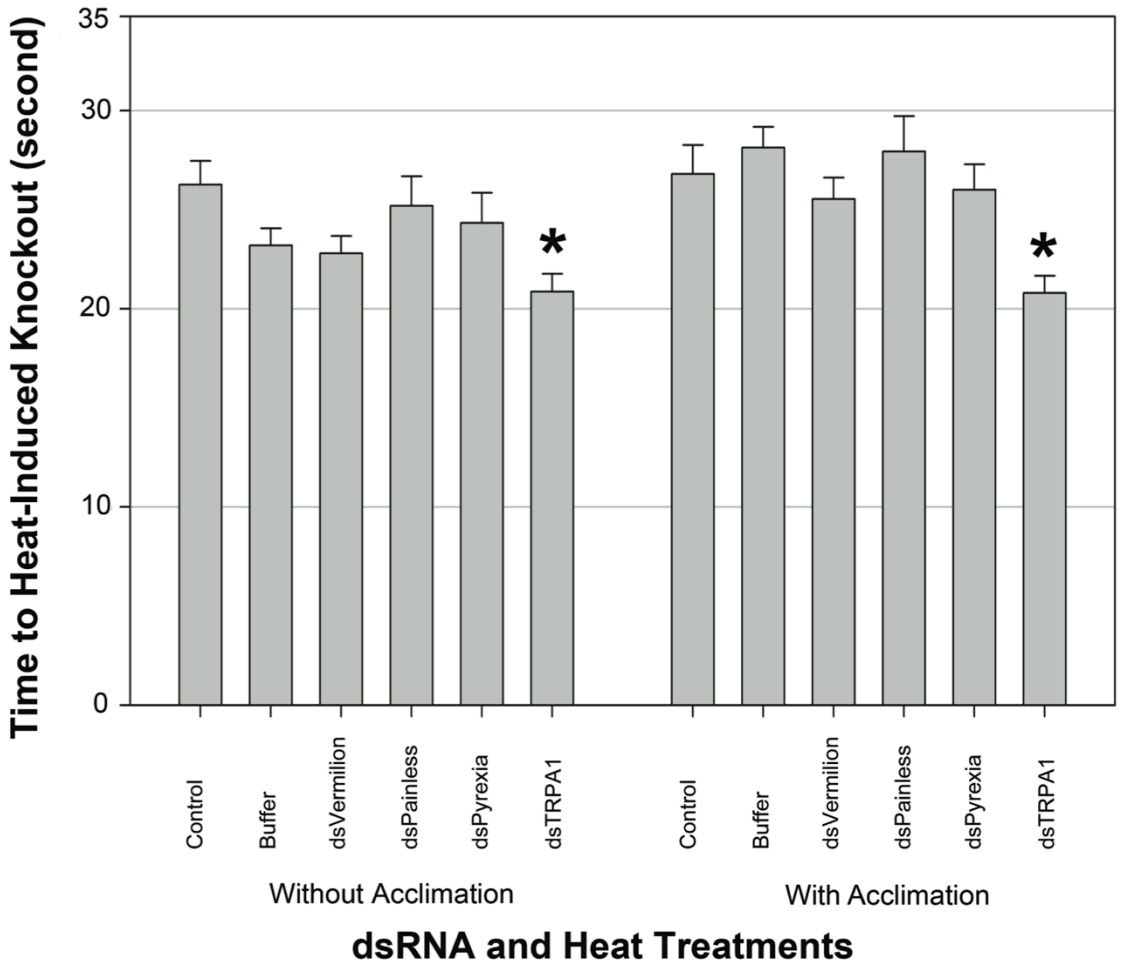

Fig. 2.7. Time to heat-induced knockout on the 52 °C hot plate either with or without a 10 min. period of acclimation at 42 °C.

Within each acclimation category, each *dsRNA* treatment was compared to controls with pairwise *t*-test. * indicates $p < 0.05$. The sample size for each bar is 30.

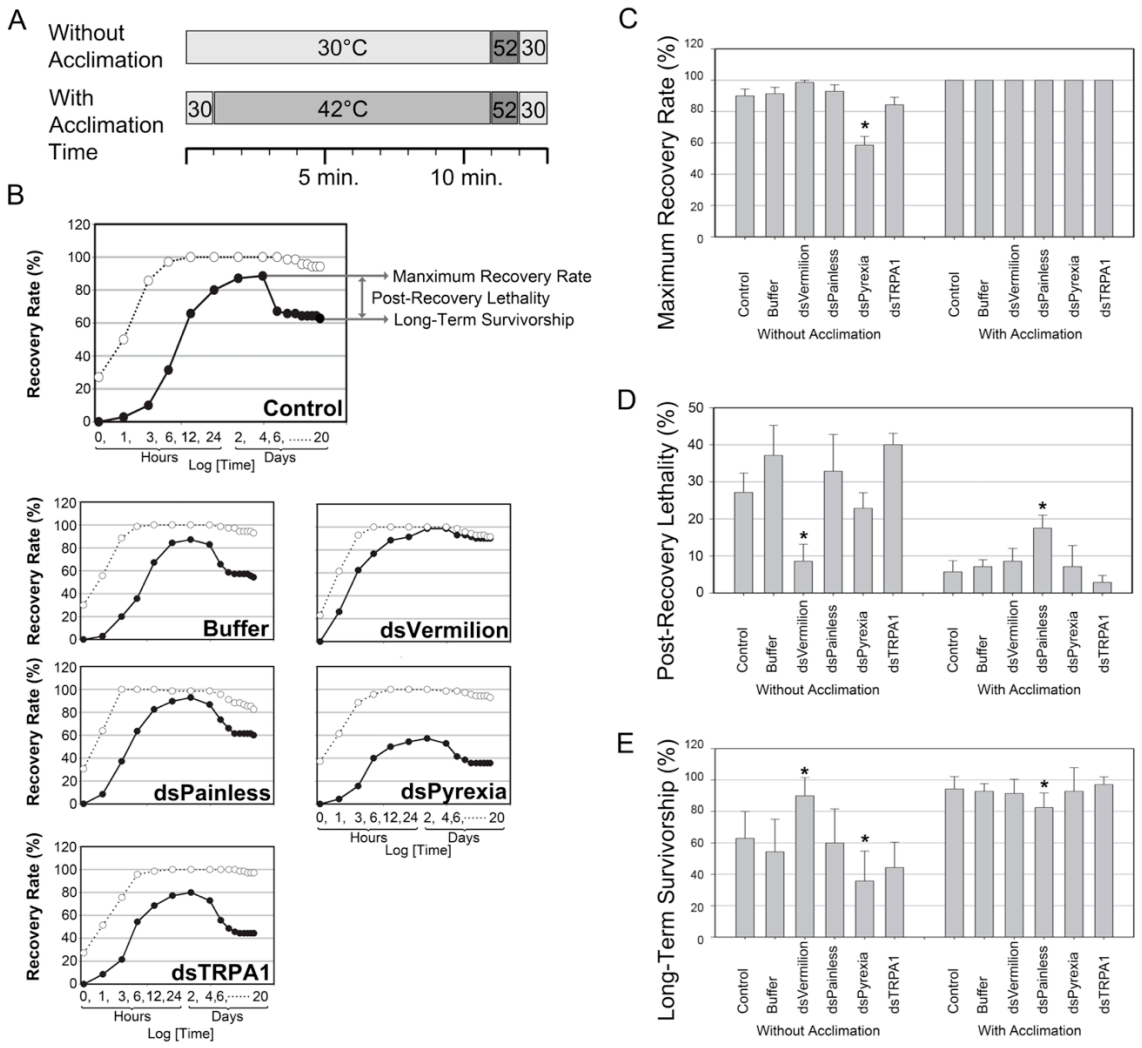

Fig. 2.8. Thermal acclimation effects on recovery rate, lethality and long-term survivorship.

A. Schematic representation of the two treatments. B. Recovery rate over time. Solid line is without acclimation, dashed with acclimation. Each line represents the mean of 70 females. C. - E. Means and standard errors of Maximum Recovery Rate (C), Long-Term Survivorship (D), and Post-Recovery Lethality (E); see text for definitions. Each *dsRNA* treatment was compared to the control group by pairwise *t*-test. * indicate $p < 0.05$.

Figure 2.9. Painless DNA – Amino Acid sequences

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    M  G  G  R  S  K  K  M  D  L  V  R  T  N  S  I  C  P  P  P  E  T  V  L  L
1   ATGGGTGGCAGAAGTAAGAAGATGGATTTGGTGCGGACTAACAGTATTTGTCCTCCACCGGAAACCGTTCTTCTA

    E  S  V  K  K  N  D  T  E  S  M  K  T  V  I  K  A  N  P  S  I  L  Q  H  L
76  GAAAGCGTGAAGAAGAACGACACAGAATCTATGAAAACAGTAATAAAAGCAAACCCATCTATTTTGCAACATCTG

                ANK1
    Y  P  Y  T  S  Q  T  I  L  S  L  A  C  S  E  P  G  V  A  P  D  T  V  N  Q
151 TACCCCTACACATCTCAAACCATCTTATCACTGGCTTGTTCCGAACCTGGCGTTGCTCCTGACACCGTCAACCAA

                                        ANK2
    L  I  E  L  G  A  D  I  E  G  T  P  Q  W  K  P  L  H  L  S  A  D  N  P  N
226 CTGATTGAATTAGGAGCAGACATCGAGGGAACCCCACAGTGGAAACCACTGCACCTATCAGCCGACAATCCCAAC

                                                                    ANK3
    I  L  I  L  E  V  V  I  K  H  L  K  P  G  Q  I  N  E  K  W  N  G  N  T  A
301 ATCCTAATTCTAGAAGTCGTCATCAAACACCTCAAACCAGGCCAAATCAATGAAAAATGGAACGGCAACACCGCC

    L  H  T  L  I  K  G  E  K  I  K  K  D  E  E  N  F  C  R  H  V  E  L  L  L
376 CTACATACGCTTATCAAAGGAGAAAAAATCAAAAAGGACGAAGAAAATTTCTGTCGGCACGTGGAGCTTCTCTTG

                                        ANK4
    Q  S  G  I  D  V  N  Q  G  D  G  K  N  L  T  A  I  F  W  A  A  K  Y  G  Y
451 CAGTCTGGAATTGATGTTAATCAGGGTGACGGCAAGAATTTAACCGCGATTTTTTGGGCCGCCAAGTACGGTTAT

    K  K  I  V  K  V  I  L  E  E  S  V  L  H  V  D  L  D  T  C  S  L  R  D  K
526 AAAAAAATTGTAAAAGTTATACTTGAGGAGAGTGTGCTTCATGTCGATCTGGACACTTGCAGTTTGCGCGATAAA

    T  A  R  D  L  I  N  E  K  G  L  Y  E  G  P  L  P  E  R  I  L  Y  Q  V  P
601 ACTGCAAGGGATTTAATAAATGAGAAAGGGTTGTACGAGGGGCCTTTGCCGGAACGTATCCTGTATCAAGTCCCC

    K  D  K  I  F  G  L  I  K  E  G  K  E  D  E  F  I  S  Y  F  E  T  L  S  L
676 AAAGACAAAATTTTCGGGTTGATTAAGGAAGGAAAAGAGGACGAGTTTATCTCTTATTTTGAGACATTGTCCTTG

                                        ANK5
    N  N  P  S  D  F  V  N  A  D  D  G  A  S  T  L  L  Q  Y  S  C  E  R  G  L
751 AATAATCCCTCCGACTTTGTTAATGCAGACGACGGTGCCTCAACACTGTTACAGTACAGTTGCGAAAGGGGGTTA
```

A K V V E Y L L D K G A N V N L V T K N Q R K P I

826 GCAAAAGTCGTAGAATATCTTTTAGACAAGGGGGCCAACGTCAATCTTGTCACAAAAAATCAAAGGAAGCCGATT

D I V A D I G Y F E I F E L L F K C P D L E L S I

901 GATATTGTCGCAGATATTGGCTACTTTGAAATTTTTGAATTGCTATTCAAATGCCCTGACTTAGAATTATCCATA

D T L C N L L K H S N S P K F G K I N H E K C C K

976 GACACACTTTGTAACCTACTAAAACACTCAAACTCTCCAAAATTTGGCAAAATCAATCACGAAAAGTGTTGCAAA

ANK6 ***RNAiF***

L L L D K L G N R R P P I D I N E V D H L N N I P

1051 TTGCTATTAGATAAACTGGGTAATAGACGTCCTCCTATTGATATCAATGAAGTGGACCATTTAAACAA**CATCCCT**

L H Y S L R Y C D T T T T Q K L L Q L G A S L A Y

1126 **CTTCACTACTCCC**TCCGGTATTGCGATACAACAACCACACAAAAATTACTGCAACTTGGAGCTTCTCTGGCTTAT

K N E F G S M P I Q D I K P E V L E N H L D N C V

1201 AAAAACGAATTCGGTTCTATGCCAATCCAAGACATCAAACCGGAAGTGCTCGAGAACCATCTCGACAACTGTGTC

T F D L K N A E K R D F E V V F D Y Q T L L P P R

1276 ACATTTGATCTCAAAAACGCCGAGAAACGCGATTTTGAAGTAGTCTTCGACTACCAGACGCTGCTACCCCCGAGA

K R K R F K Y E E V D P E F L A T N N V K P E T E

1351 AAACGCAAGCGATTCAAATACGAAGAGGTGGACCCTGAATTTTTGGCCACAAACAATGTAAAACCTGAAACTGAA

V I A Y M S K A P E F R A L L K H P L I V S F L F

1426 GTTATAGCCTACATGAGCAAAGCTCCAGAATTTCGAGCCTTGCTCAAGCACCCCCTTATTGTCAGTTTCCTCTTT

TM1

M K W H Q I R L L F Y T N L V F Y I C F V L S L V

1501 ATGAAATGGCACCAAATCCGCTTACTGTTTTACACAAATCTGGTGTTCTATATTTGCTTTGTGCTGTCTTTAGTT

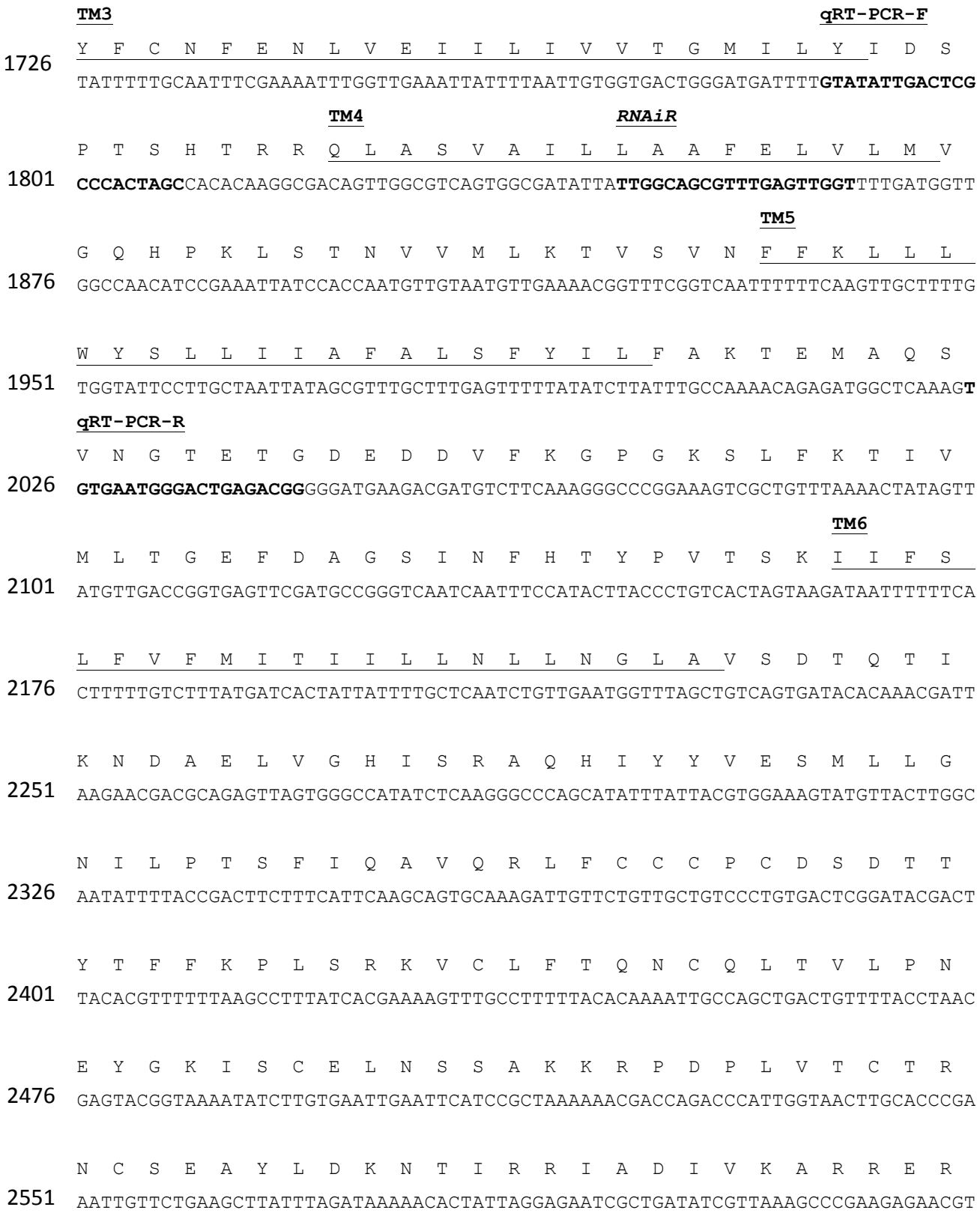
TM2

V Y I F T H Y A N F D R T Q S D Y C L I F G K F S

1576 GTCTACATTTTTACGCATTATGCTAATTTCGACCGGACGCAGTCGGATTATTGTCTCATTTTTGGCAAATTTTCG

W F T L N L T F W V L V L R E V F Q V A V A P R K

1651 TGGTTCACTCTAAATTTAACATTTTGGGTTTTGGTGTTACGTGAGGTTTTCCAAGTCGCGGTGGCGCCTCGAAAA


TM3
qRT-PCR-F
Y F C N F E N L V E I I L I V V T G M I L Y I D S
1726
TATTTTGCAATTTCGAAAATTTGGTTGAAATTATTTTAATTGTGGTGACTGGGATGATTTTGTATATTGACTCG
TM4
RNAiR
P T S H T R R Q L A S V A I L L A A F E L V L M V
1801
CCCACTAGCCACACAAGGCGACAGTTGGCGTCAGTGGCGATATTATTGGCAGCGTTTGAGTTGGTTTTGATGGTT
TM5
G Q H P K L S T N V V M L K T V S V N F F K L L L
1876
GGCCAACATCCGAAATTATCCACCAATGTTGTAATGTTGAAAACGGTTTCGGTCAATTTTTTCAAGTTGCTTTTG
W Y S L L I I A F A L S F Y I L F A K T E M A Q S
1951
TGGTATTCCTTGCTAATTATAGCGTTTGCTTTGAGTTTTTATATCTTATTTGCCAAAACAGAGATGGCTCAAAGT
qRT-PCR-R
V N G T E T G D E D D V F K G P G K S L F K T I V
2026
GTGAATGGGACTGAGACGGGGGATGAAGACGATGTCTTCAAAGGGCCCGGAAAGTCGCTGTTTAAAACTATAGTT
TM6
M L T G E F D A G S I N F H T Y P V T S K I I F S
2101
ATGTTGACCGGTGAGTTCGATGCCGGGTCAATCAATTTCCATACTTACCCTGTCACTAGTAAGATAATTTTTTCA
L F V F M I T I I L L N L L N G L A V S D T Q T I
2176
CTTTTTGTCTTTATGATCACTATTATTTTGCTCAATCTGTTGAATGGTTTAGCTGTCAGTGATACACAAACGATT
K N D A E L V G H I S R A Q H I Y Y V E S M L L G
2251
AAGAACGACGCAGAGTTAGTGGGCCATATCTCAAGGGCCCAGCATATTTATTACGTGGAAAGTATGTTACTTGGC
N I L P T S F I Q A V Q R L F C C C P C D S D T T
2326
AATATTTTACCGACTTCTTTCATTCAAGCAGTGCAAAGATTGTTCTGTTGCTGTCCCTGTGACTCGGATACGACT
Y T F F K P L S R K V C L F T Q N C Q L T V L P N
2401
TACACGTTTTTTAAGCCTTTATCACGAAAAGTTTGCCTTTTTACACAAAATTGCCAGCTGACTGTTTTACCTAAC
E Y G K I S C E L N S S A K K R P D P L V T C T R
2476
GAGTACGGTAAAATATCTTGTGAATTGAATTCATCCGCTAAAAAACGACCAGACCCATTGGTAACTTGCACCCGA
N C S E A Y L D K N T I R R I A D I V K A R R E R
2551
AATTGTTCTGAAGCTTATTTAGATAAAAACACTATTAGGAGAATCGCTGATATCGTTAAAGCCCGAAGAGAACGT

```
      E  E  Y  P  T  V  T  N  L  K  Q  L  Y  G  E  I  V  S  I  K  A  K  L  D  Q
2626  GAAGAGTATCCTACAGTTACTAATCTAAAGCAGTTATATGGAGAAATTGTGAGCATAAAAGCGAAGCTCGATCAA

      I  L  G  S  L  S  T  N  Q  N  A  L  *
2701  ATTTTGGGAAGTCTCTCGACAAATCAAAACGCTTTATGA
```

Figure 2.10. Pyrexia DNA – Amino Acid sequences

```
    M  S  L  R  H  E  R  P  G  K  T  I  E  E  S  T  V  R  W  N  G  D  I  S  L
1   ATGTCGCTGCGGCATGAAAGACCTGGAAAAACCATTGAAGAATCGACGGTTCGATGGAACGGCGACATCTCTCTG

    D  M  T  D  E  M  G  S  I  S  S  E  D  E  T  G  S  E  Y  D  G  E  Q  R  S
76  GATATGACAGATGAGATGGGCAGCATCTCCAGCGAGGATGAGACGGGGTCTGAGTACGATGGGGAACAAAGAAGT

    R  G  K  S  V  L  E  I  W  D  D  D  Y  I  Q  A  R  L  Q  A  S  /I  L  P  D
151 AGAGGGAAGTCAGTTTTAGAGATATGGGACGATGACTACATCCAGGCTAGACTACAGGCGAGT/ATATTGCCTGAT
                                                                  Intron1(41bp)

    A  E  D  I  I  E  M  I  E  Q  D  N  T  E  T  I  F  T  K  N  P  T  S  L  L
226 GCCGAAGATATAATCGAGATGATTGAACAAGACAATACGGAGACGATTTTTACGAAAAATCCGACGAGTCTACTG

    L  I  A  T  W  L  Q  K  E  K  V  L  Q  E  V  L  E  K  G  V  S  L  Q  A  V
301 CTTATCGCTACGTGGTTGCAAAAGGAGAAGGTTCTTCAGGAGGTTTTGGAGAAGGGAGTTTCTCTGCAAGCCGTA
          ANK1
    D  G  E  G  R  /  S  A  L  H  L  A  A  C  T  G  N  I  D  C  I  K  L  L  L  Q
376 GACGGAGAAGGACG/ATCGGCGCTCCACTTAGCCGCCTGCACCGGCAACATCGACTGCATCAAACTCCTTCTCCAG
                Intron2(2993bp)
                                        ANK2
    H  G  A  E  I  S  A  R  D  A  L  N  R  A  T  P  L  H  C  A  A  S  K  G  H
451 CACGGCGCTGAAATCAGCGCCCGCGACGCCCTCAACCGCGCCACCCCTCTCCACTGCGCCGCCAGCAAAGGCCAC
                                                          ANK3
    L  S  A  V  K  L  L  I  R  H  G  A  D  V  N  A  G  L  D  N  K  S  P  L  H
526 CTATCCGCCGTCAAACTCCTCATCCGGCACGGCGCCGACGTCAACGCCGGCCTCGACAACAAAAGCCCTCTTCAC

    Y  A  V  Q  S  L  A  I  D  C  V  K  E  L  L  E  N  N  A  I  P  N  T  S  Q
601 TACGCCGTCCAAAGCCTTGCAATCGACTGCGTGAAGGAACTCCTCGAGAACAACGCCATCCCCAACACCTCCCAA
       ANK4
    V  Y  S  E  T  P  L  H  V  A  A  A  L  G  A  P  E  I  V  K  L  L  L  D  H
676 GTATACAGCGAGACGCCACTCCACGTCGCTGCGGCCCTAGGCGCCCCCGAAATCGTCAAACTCCTCCTGGACCAC
                                    RNAiF ANK5
    G  A  A  V  N  V  Q  C  G  T  D  K  L  T  P  L  H  L  A  A  E  D  S  D  A
751 GGCGCCGCCGTCAACGTCCAATGCGGCACCGATAAACTCACCCCTCTGCACCTGGCCGCGGAGGACAGCGACGCC
```

ANK6

E S A R L L I D A G A Q L T S E N H K K Q T P L H

826 GAAAGCGCCCGCCTCCTCATCGACGCCGGTGCCCAACTCACCAGCGAAAATCACAAAAAGCAAACGCCGCTTCAT

L A A L S Q C S E T L E L L L A R G C N P N A R D

901 TTGGCCGCGTTGTCGCAATGCTCGGAAACACTCGAATTGCTCCTAGCGAGGGGGTGCAACCCCAATGCGAGAGAT

ANK7

A D G R T P L H G A I V K V S R S C E C V R L L L

976 GCTGATGGGCGGACGCCCCTCCATGGGGCCATAGTCAAGGTTTCACGCTCGTGTGAATGTGTCAGACTTTTGCTG

ANK8

K A G A D V N R Q D S F G Y T P L H L A A L N E F

1051 AAAGCGGGAGCTGATGTCAATCGGCAAGATTCCTTCGGGTACACTCCGCTACATCTGGCGGCGCTTAATGAATTC

S N C V M M L L N H G G D V T V R T N G G V S V L

1126 TCAAATTGTGTCATGATGCTACTCAATCATGGGGGCGATGTGACAGTGCGGACGAACGGAGGAGTCTCCGTTTTG

S F I T R K T P D V I P R Y I S K F D S S I K I N

1201 AGTTTCATTACGAGGAAAACACCCGATGTTATCCCGAGATATATTTCCAAGTTTGATTCTTCGATTAAAATCAAC

D H E I G D V D C E L K L D F R I L V P T M G H Q

1276 GACCATGAAATAGGAGACGTCGACTGTGAACTCAAGCTTGATTTCAGGATTCTGGTCCCGACTATGGGGCACCAA

E T E L L L N F I E V G H R E V L K H P L C E T F

1351 GAAACCGAACTTTTGCTCAACTTCATCGAAGTCGGGCATAGGGAAGTGCTCAAGCACCCACTCTGCGAAACTTTC

TM1

L F L K W R R I R K F F L F S L F Y H S L F V L L

1426 CTTTTCCTCAAGTGGCGAAGAATCCGGAAATTCTTCCTCTTCAGTCTCTTCTATCACAGTCTTTTCGTCTTGTTG

RNAiR

F S I Y T I G V Y I Q D C P S F R A L L T R P C R

1501 TTCTCGATTTACACCATCGGGGTGTACATACAAGACTGTCCTTCGTTTCGCGC**TCTCCTAACTCGTCCGTGTC**GA

TM2

V P Q Y Y N I I G Y I L L V F N F M F L A K E L F

1576 GTTCCACAGTATTACAATATCATTGGTTACATTCTCCTTGTATTCAATTTTATGTTTCTGGCGAAGGAACTGTTC

TM3

Q I C H S W R S Y I Q Q W E N W L Q W L I I I S V

1651 CAAATTTGCCACTCTTGGCGCAGTTACATCCAACAGTGGGAGAACTGGTTGCAATGGCTCATCATAATCAGTGTT

F C C V Q P S L D N D M D I R Y K V M R W Q H H V

1726 TTCTGCTGCGTGCAACCCAGTTTAGACAACGACATGGACATCAGATACAAAGTGATGCGGTGGCAACACCACGTG

TM4

A A V G I F L A W V E L M M I V G R F P I F G L Y

1801 GCCGCCGTAGGGATTTTCCTGGCCTGGGTCGAGCTCATGATGATAGTTGGACGATTCCCAATTTTCGGACTCTAC

TM5

I Q M F T T V A V N F I K F V I A Y F C L L L A F

1876 ATCCAAATGTTCACCACAGTCGCTGTCAACTTCATAAAATTCGTGATCGCTTATTTCTGCCTTCTCCTAGCTTTC

A F S F G V L F A K Y K S F K L L K W I I I K V L

1951 GCCTTCAGTTTCGGCGTGCTTTTCGCCAAATATAAATCGTTCAAACTTTTGAAATGGATCATTATTAAAGTCCTA

V M M S G E L E Y E D I F Y D E E A P I Q Y D Y T

2026 GTAATGATGTCGGGTGAATTAGAATATGAAGACATTTTCTACGACGAGGAAGCTCCGATTCAGTACGACTACACC

qRT-PCR-F **TM6**

S Q F V F L A Y V I L V T I I L A N L L V G L A V

2101 TCC**CAGTTCGTGTTTCTCGCCTA**CGTTATCCTAGTTACAATCATTTTGGCCAATCTTTTGGTCGGGTTAGCTGTC

S D I Q G L Q Q S A G L D R L V R Q A E L V A H L

2176 AGTGACATACAGGGTTTGCAACAAAGTGCAGGTCTTGACAGATTGGTCAGACAAGCCGAGCTCGTGGCTCATCTC

E S M L F S R L L T C I P H K L M H F F H K K A L

2251 GAGTCGATGCTTTTTTCCCGCTTGTTGACTTGTATCCCGCACAAACTGATGCATTTTTTTCATAAGAAAGCGCTT

L L K S Q Y H W A L Y I K P N D P R E E R I P K D

2326 CTTTTGAAATCGCAATACCACTGGGCGCTTTATATCAAACCGAACGATCCGCGAGAGGAACGCATACCCAAGGAT

L I K N I Y Q L V A E R K E K P R K K R R S N K I

2401 TTGATTAAAAATATTTACCAATTGGTGGCGGAGAGAAAAGAAAAGCCGCGGAAGAAACGAAGGAGCAATAAAATT

K S D F V S P P M S R L N S I S D S Y G G A D K Q

2476 AAATCCGATTTTGTTTCGCCGCCAATGTCGAGACTGAATTCGATTTCTGATTCGTATGGAGGTGCTGATAAACAA

qRT-PCR-R

V A L K C E L E E I Q K E F A E F T R T F R E K M

2551 GTGGCTTTGAAGTGCGAGTTGGAGGAGATTCAAAAGGAGTTTGCCGAATTTACCCGGACGTTC**AGGGAGAAAATG**

E G I T N Q I K N T K S *

2626 **GAGGGCATAA**CGAATCAAATTAAAAATACAAAGAGTTAA

Figure 2.11. TRPA1 DNA – Amino Acid sequences

```
    M  L  P  S  S  V  K  V  H  R  L  S  N  A  G  K  P  P  E  D  N  G  G  I  C
1   ATGCTGCCATCTTCCGTCAAAGTGCACAGATTGAGCAACGCCGGAAAACCTCCGGAGGATAACGGCGGCATCTGT

    L  M  T  E  S  P  F  R  I  L  R  /V  A  E  C  G  N  L  E  T  F  Q  R  L  Y
76  CTCATGACGGAGAGTCCTTTCCGGATATTGAGG/GTGGCGGAATGCGGCAACTTGGAGACTTTTCAGCGACTGTAT
                                  Intron(11724bp)
                                 ANK1
    F  A  D  P  T  R  L  S  I  K  D  S  R  G  R  T  A  A  H  Q  A  A  A  K  N
151 TTCGCTGATCCGACCAGGCTTAGTATTAAGGACAGCAGAGGCAGGACTGCAGCTCATCAAGCAGCAGCAAAGAAC
                                                                ANK2
    R  I  T  I  L  Q  F  I  L  S  Q  G  G  D/ L  N  N  Q  D  N  A  G  N  T  P
226 AGAATTACCATTTTACAATTTATCTTGTCACAAGGAGGCG/ATCTTAATAATCAGGATAATGCCGGGAATACGCCT
                                        Intron(48bp)

    L  H  V  A  V  E  H  E  S  L  D  A  V  D  F  L  L  Q  A /G  V  K  T  N  I
301 CTTCATGTCGCTGTGGAACACGAGTCGTTGGATGCGGTGGATTTTCTCCTTCAAGC/GGGCGTAAAGACGAATATA
                                                    Intron(3583bp)
          ANK3
    L  N  D  K  K  Q  A  A  I  H  L  V  T  E  L  N  K  V  S  V  L  E  V  M  G
376 TTAAACGACAAGAAACAAGCCGCCATCCATTTGGTCACCGAGCTTAACAAGGTGTCCGTGCTGGAGGTCATGGGC
                                       ANK4
    K  H  K  D  K  I  D  I  L  Q  G  G  E  H  G  R  T  A  L  H  I  A  A  I  Y
451 AAGCATAAGGACAAGATAGACATCCTGCAAGGTGGGGAACATGGCAGGACTGCTCTGCATATCGCTGCCATTTAC
                                                                ANK5
    D  H  E  E  C  A  R  I  L  /I  S  V  F  D  A  C  P  R  R  P  C  N  N  G  Y
526 GACCACGAGGAGTGTGCCAGGATATTG/ATCTCCGTCTTCGACGCGTGCCCTCGCCGCCCTTGCAACAACGGCTAC
                                  Intron(2479bp)

    Y  P  I  H  E  A  A  K  N  A  S  S  K  T  L  E  I  F  L  Q  W  G  E  S  R
601 TACCCTATTCACGAAGCCGCCAAAAACGCCTCCTCTAAAACCCTCGAGATTTTCCTACAGTGGGGTGAATCACGC
                                       ANK6
    G  C  T  R  E  E  M  I  S  F  Y  D  S  E  G  N  V  P  L  H  S  A  V  H  G
676 GGATGTACACGTGAAGAAATGATTTCGTTTTACGACTCCGAAGGTAACGTTCCGTTACACTCTGCTGTTCACGGT
```

ANK7

G D I K A V E L C L R S G A K I S T Q Q H D L S T

751 GGAGATATCAAAGCAGTGGAATTGTGTTTACGATCAGGTGCAAAAATAAGCACCCAACAACACGACCTCTCAACC

P V H L A C A Q G A T D I V K L M F K M Q P E E K

826 CCTGTACACTTAGCGTGTGCTCAAGGAGCTACAGACATCGTCAAACTCATGTTCAAGATGCAACCGGAAGAGAAA

ANK8

L P C L A S C D V Q K /M T P L H C A A M F D H P E

901 TTACCCTGTTTGGCATCATGTGACGTCCAGAAG/ATGACCCCACTTCACTGTGCTGCCATGTTCGACCATCCTGAG

Intron(259bp)

ANK9

I V E F L I N E G A D I N P M D K E K R S P L L L

976 ATCGTCGAGTTTTTGATTAATGAAGGCGCTGATATCAACCCGATGGACAAGGAGAAGCGATCACCTCTACTTCTA

A A L R G G W R T V H V L I R L G A D I N V K D V

1051 GCTGCACTCAGAGGGGGTTGGAGGACAGTTCATGTGCTGATTAGACTAGGGGCCGATATTAACGTTAAAGATGTT

N R R N V L H L V V M N G G R L E Q F A S E V S K /

1126 AATCGACGGAATGTTTTACATCTGGTGGTGATGAATGGAGGGCGGTTGGAACAATTTGCGTCGGAAGTTAGCAAG/

Intron(689bp)

ANK10

A K S Q T S L L Q L L N E K D I N G C S P L H Y A

1201 GCCAAATCGCAAACGAGTCTCCTCCAACTGCTCAACGAAAAAGACATTAACGGCTGTTCGCCTCTTCACTACGCC

ANK11

S R E G H I R S L E N L I R L G A T I N L K N N N

1276 AGTAGAGAGGGCCATATCAGAAGTTTAGAAAATTTGATTCGACTTGGAGCCACCATCAACCTCAAAAACAACAAC

N E S P L H F A A R / Y G R Y N T V R Q L L D S E K

1351 AACGAAAGTCCTTTACATTTTGCCGCTAG/ATATGGGCGCTACAACACAGTCCGCCAACTCCTGGACTCAGAAAAA

Intron(52bp)

ANK12

G T F I I N E S D G E G L T P L H I A S K Q G H T

1426 GGCACTTTCATAATCAACGAAAGCGACGGCGAGGGTCTCACCCCCCTCCACATCGCTTCCAAGCAAGGCCACACC

ANK13

R V V Q L L L N R G A L L H R D H N G R N P L H L

1501 CGCGTTGTCCAGCTCCTCCTCAACCGTGGCGCTCTTCTCCATCGTGACCACAACGGACGCAACCCCCTCCACCTT

A A M N G Y T Q T I E L L L S V H S H L L D Q T D

1576 GCTGCCATGAACGGCTACACCCAAACCATCGAGCTCCTCCTCTCTGTCCACTCCCACCTCCTGGACCAGACTGAC

ANK14

K D G N T A L H L A T M E N K P N A I A L L L S M

1651 AAAGACGGCAACACGGCCTTACATTTAGCCACAATGGAGAATAAACCAAACGCTATCGCTCTCCTTCTCTCAATG

ANK15

N C K L L Y N Q M E M S A I D Y A I Y Y K F P E A

1726 AACTGCAAACTTTTGTACAACCAGATGGAGATGAGTGCCATTGACTACGCCATCTATTACAAATTTCCCGAAGCT

A L A M V T H E D R A E E V M A L K S S K H P Y V

1801 GCCTTGGCCATGGTCACGCATGAGGACCGGGCCGAGGAAGTTATGGCCTTAAAATCGAGCAAACATCCATATGTC

qRT-PCR-F

T L A L I A S M P R V F E A V Q D K C I T K A N C

1876 ACTCTTGCACTGATTGCTTCGATGCCACGAGTGTTTGAAGCTGTTCAAGACAAGT**GTATCACCAAAGCCAACTGC**

RNAiF

K K D S K S F Y /I K Y N F S A L Q C S Q F Y A D M

1951 AAGAAGGACTCAAAGTCGTTCTAT/ATTAAATACAACTTTTCGGCCTTGCAA**TGCTCACAGTTTTACGCCGA**TATG

Intron(46bp)

qRT-PCR-R

D H K T G D A L A I S K P I P L P A L N /A M V S H

2026 GACCATAAGACAGGAGATGCTCTTGCTATTTCTAAACCTATTCCTTTGCCGGCTTTAAAC/G**CAATGGTTTCGCAC**

Intron(52bp)

G R V E L L A H P L S Q K Y L Q M K W N S Y G K Y

2101 **GGTAGA**GTTGAACTGTTGGCCCACCCATTGAGCCAAAAGTACCTCCAAATGAAATGGAACTCGTACGGAAAGTAC

TM1

F H L T N V L F Y S I F L T F V T C F A Y E I M R

2176 TTCCATCTAACTAACGTCCTATTCTACAGTATTTTCCTAACTTTTGTGACGTGTTTCGCGTACGAAATCATGCGA

H E D Q I I T Y N A T N L T H D /E Y V N F S K A N

2251 CACGAGGATCAAATCATAACTTACAATGCCACCAACTTGACACATGAT/GAATACGTTAATTTTAGCAAAGCCAAT

Intron(48bp)

TM2

I L N V K I T P M M Y M S A L A I I T Y I I L N T

2326 ATACTAAACGTCAAGATAACACCAATGATGTACATGAGTGCCCTTGCTATCATCACTTACATTATCTTGAACACC

TM3

I R E M V Q V Y Q Q K F M Y F L D P N N L V T W V

2401 ATACGCGAAATGGTTCAAGTGTACCAACAAAAATTTATGTATTTCCTGGATCCTAACAATTTAGTGACTTGGGTA

RNAiR **TM4**

L Y T C A V V M V F P I F W G T M Y E L Q F S C A

2476 CTTTATACATGTGCAGTAGTTATGGTTT**TTCCGATTTTTTGGGGCA**CCATGTACGAGCTCCAATTCTCGTGTGCT

S V T V F L S W F N L L L L L Q R F D Q V G I Y V

2551 TCAGTCACTGTTTTCCTGAGTTGGTTCAACCTTCTCCTCCTGTTGCAACGTTTCGACCAAGTGGGGATTTACGTA

TM5

V M F L E I L Q T L I K V L L V F S I L I I A F G

2626 GTTATGTTTCTCGAGATCTTGCAAACACTTATAAAAGTACTCCTCGTGTTCTCCATCCTGATTATTGCATTTGGT

L A F Y I L L S R /G D H L S F K T I P M S L V R T

2701 CTTGCGTTCTATATTTTATTGTCACGG/GGTGACCATCTTAGCTTCAAAACGATACCAATGAGTCTAGTTCGGACG

Intron(50bp)

F S M M L G E I D F L G T Y V K P Y Y L T T E D E

2776 TTTTCAATGATGTTGGGCGAGATTGATTTTTTGGGGACGTACGTCAAGCCGTACTACTTGACCACGGAAGACGAG

TM6

K S F L P F P L P A F F I L G L F M V L M P I L L

2851 AAGTCATTTTTGCCCTTCCCCCTTCCTGCGTTTTTCATTTTGGGGCTCTTTATGGTACTGATGCCCATCCTATTG

M N L L I G L A V G D I E S V R R N A Q L K R L A

2926 ATGAATTTGCTCATTGGTTTGGCCGTTGGTGACATAGAATCAGTCAGGAGGAACGCTCAGTTGAAACGATTGGCA

M Q /V V L H T E L E R K L P K M L L E R V D K C E

3001 ATGCAG/GTGGTTTTGCACACGGAACTCGAGCGCAAACTCCCTAAAATGCTCCTGGAACGTGTTGACAAGTGCGAA

Intron(51bp)

L I E Y P N D T K C K L G F F D S I L R K W F G N

3076 CTGATCGAATATCCCAACGATACCAAGTGCAAGCTGGGTTTCTTCGACTCCATCTTGCGCAAATGGTTCGGGAAC

P F S D E G/ V T T D T R S Q Q D G G V M G/ L D M A

3151 CCGTTTTCGGACGAGG/GAGTCACCACAGACACAAGATCACAGCAAGATGGCGGCGTCATGG/GTTTGGACATGGCG

Intron(124bp) Intron(98bp)

```
     M  E  G  V  E  D  Y  V  V  N  E  L  D  K  T  K  R  K  L  K  E  I  T  T  A
3226 ATGGAAGGTGTCGAGGATTACGTTGTGAACGAACTCGACAAAACCAAGAGAAAGTTGAAGGAAATTACCACAGCT

     L  E  T  Q  Q  Q  F  L  R  L  I  V  Q  K  M  E  I  K  T  E  A  D  D  V  D
3301 TTGGAGACACAGCAGCAGTTTTTGAGGCTGATTGTGCAAAAAATGGAAATTAAGACGGAAGCTGACGATGTTGAC

     E  G  V  S  P  N  D  L  K  P  I  T  G  H  A  S  K  W  T  S  P  K  I  R  K
3376 GAGGGGGTTTCCCCAAATGATCTTAAACCCATCACGGGACATGCGAGCAAGTGGACTTCGCCCAAAATCAGGAAG

     K  L  R  S  V  V  S  F  N  N  K  G  S  S  T  *
3451 AAGTTGCGGTCGGTGGTCAGTTTCAACAATAAAGGCAGCAGCACTTAG
```

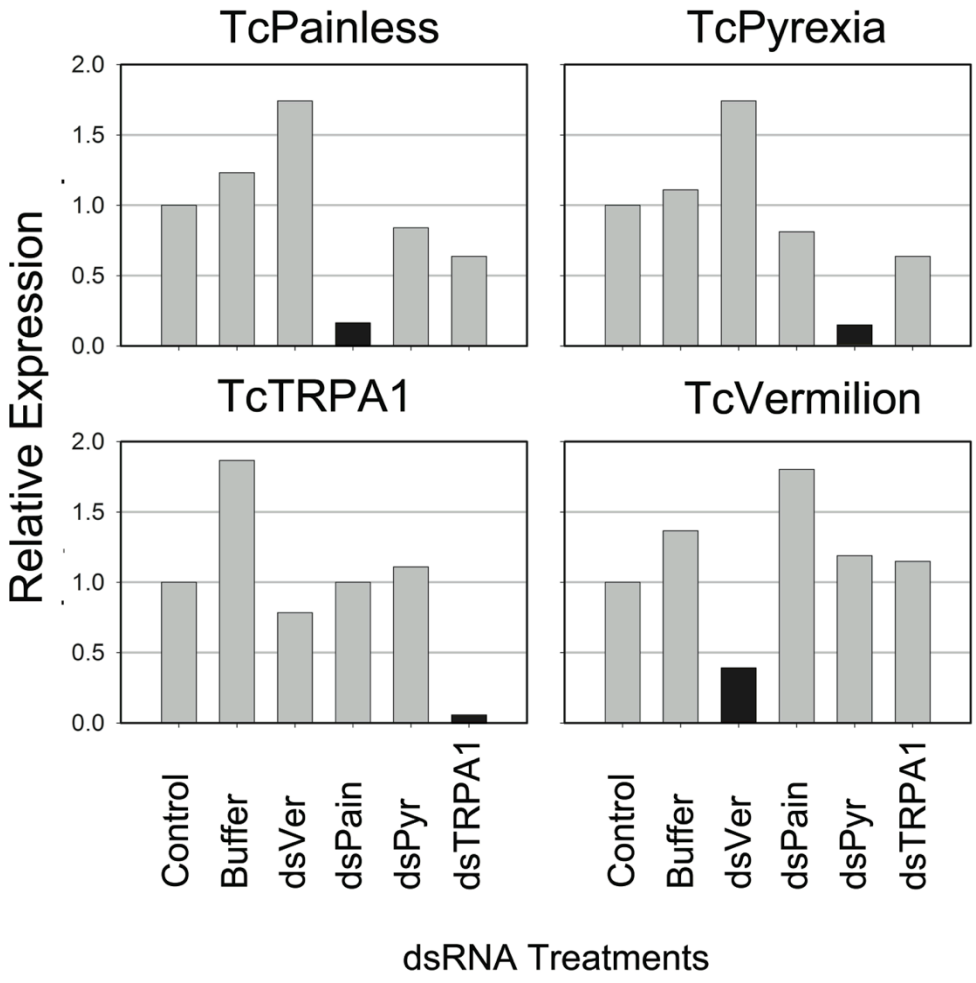


Fig. 2.S1. Effectiveness of *dsRNA* treatment.

Each bar represents two technical replications of qRT-PCR. The Ct values of different *dsRNA* treatments were normalized relative to the control group.

Table 2.1. The primers were used for *dsRNA* synthesis and RT-qPCR

	dsRNA	RT-qPCR
Painless	TAATACGACTCACTATAGGGCATCCCTCTTCACTACTCCC	GTATATTGACTCGCCCACTAGC
	TAATACGACTCACTATAGGGACCAACTCAAACGCTGCCAA	CCGTCTCAGTCCCATTCACA
Pyrexia	TAATACGACTCACTATAGGGCCGATAAACTCACCCCTCTG	CAGTTCGTGTTTCTCGCCTA
	TAATACGACTCACTATAGGGACACGGACGAGTTAGGAGA	TTATGCCCTCCATTTTCTCCCT
TRPA1	TAATACGACTCACTATAGGGTGCTCACAGTTTTACGCCGA	GTATCACCAAAGCCAACTGC
	TAATACGACTCACTATAGGGTGCCCCAAAAAATCGGAA	TCTACCGTGCGAAACCATTG
Vermilion	TAATACGACTCACTATAGGGGAGCAAATCGCCAAGTCGG	TTCTCACACAAAGCCCTGC
	TAATACGACTCACTATAGGGCCTGGGTTCGTCCCTGTAA	GCGAGTCTATGTCCATCAAAAG
RPS3		ACCGTCGTATTCGTGAATTGAC
		ACCTCGATACACCATAGCAAGC

Table 2.2. Sample size for each temperature comparison and *dsRNA* treatment.

Six beetles were used per replication, and 3-5 replications were run.

	30 vs. 30 °C	30 vs. 33 °C	30 vs. 36 °C	30 vs. 39 °C	30 vs. 42 °C
Control	18	18	18	24	24
Buffer	24	24	24	30	30
dsVer	18	18	18	18	18
dsPain	18	18	18	24	24
dsPyr	18	18	18	24	24
dsTRPA1	24	24	24	30	30

Table. 2.3. Exemplary movement data for one individual for each *dsRNA* treatment at 30 vs. 30 ^{o}C.

Each beetle was monitored for 15 min (900 sec).

	Combined		Control Zone			Experimental Zone			Summary	
dsRNA Treatment	Distance (cm)	Speed (cm/sec)	Distance (cm)	Time (sec.)	Speed (cm/sec)	Distance (cm)	Time (sec.)	Speed (cm/sec)	% of time	Rel. Speed
Control	200.20	0.22	109.95	351.95	0.31	90.25	548.25	0.16	39.10	52.71
Buffer	145.53	0.16	62.78	397.00	0.16	82.75	503.20	0.16	44.10	104.01
dsVer	184.92	0.21	110.17	581.78	0.19	74.75	318.42	0.23	64.63	124.00
dsPain	174.78	0.19	99.47	642.44	0.15	75.31	257.76	0.29	71.37	188.67
dsPyr	184.84	0.21	92.04	393.69	0.23	92.80	506.51	0.18	43.73	78.35
dsTRPA1	139.49	0.15	70.69	536.04	0.13	68.79	364.16	0.19	59.55	143.21

$$\text{PTS} = \left\{ \frac{\text{Time Spent in a Control Zone}}{\text{Total Time}} \right\} \text{X } 100$$

$$\text{Relative Speed} = \left\{ \frac{\text{Speed in an Experimental Zone}}{\text{Speed in a Control Zone}} \right\} \text{X } 100$$

Chapter 3 - The Role of Mechanosensory Transient Receptor Potential Channels and Insect Cytokines in Tonic Immobility in the Red Flour Beetle, *Tribolium castaneum*

Abstract

Animals have mechanosensory abilities that allow them to perceive external and internal physical stimuli, such as touch, vibration, nociception, and proprioception through specialized mechanosensors. Transient receptor potential channels (TRPs) are a family of cation channels, involved in various sensory mechanisms including mechanosensing in *Drosophila melanogaster*. When phylogenetic clusters of mechanosensory TRPs in *D. melanogaster* - seven members in TRPV, TRPN, and TRPA subfamilies - were compared to TRPs in the beetle *Tribolium castaneum,* I found one-to-one orthologous relationships in all except for *TctrpA5*, which has been likely lost in *D. melanogaster*. I then investigated the functions of the eight candidate mechanosensory TRPs in *T. castaneum* in survival, walking behavior, and tonic immobility by using RNA interference (RNAi) technique. In addition, I also examined candidate humoral factors involved in the tonic immobility, insect cytokines (ICKs). Double stranded RNA treatment of two TRPs, *nompC* (*dsnompC*) and *dstrpA5,* resulted in failure in eclosion, causing 93 % of mortality in each treatment. Surivors of *dsnompC* treatment showed defects in sclerotization of the elytra. With *dsnanchung* and *dsinactive* treatments, the adults showed defects in folding the joint between the femur and tibia of the hind legs, which caused abnormal walking behavior and reduced walking speed. Tonic immobility, a short knockout- response of the beetle to the mechanical stimulation on the ventral

surface, was sensitized with *dsnanchung*, *dsinactive*, and *dswaterwitch*. Beetles treated with *dsick2* (insect cytokine 2) also showed increased sensitivity for tonic immobility. These data suggest that *nompC* and *trpA5* are essential for adult eclosion likely through proprioception feedback. The function of *nanchung* and *inactive* may be as mechanoreceptors specialized for mechanical proprioception in the hind leg segments. In addition, *nanchung*, *inactive*, and *waterwitch* are likely involved as receptors in tonic immobility. A humoral factor *ick2* is suggested as a downstream mediator of the mechanosensing in *T. castaneum* tonic immobility.

Introduction

Organisms have abilities to detect mechanical signals from their environment through various mechanosensors that serve for accurate evaluation of touch, nociception, hearing, and proprioception. The molecular mechanism for these processes involves specialized cells in which specific membrane channels are activated when a specific physical force is applied. Transient receptor potential channels (TRPs) are a group of membrane channel functioning in mechanosensing . TRPs are six-transmembrane polypeptide subunits that make a tetramer to form a cation channel (Montell, 2005). This family of channels is further categorized into seven TRP subfamilies by its homology (Clapham, 2003). The functions of TRP channels are highly diverse, including various sensory mechanisms such as vision, taste, smell, hearing, hygrosensation, thermosensation, and mechanosensation (Mori, 1999; Kim et al., 2003; Tracey et al., 2003; Gong et al., 2004; Lee et al., 2005; Rosenzweig et al., 2005; Dhaka et al., 2006; Liu et al., 2007; Rosenzweig et al., 2008). Mechanosensory TRP channels have been

discovered in *Drosophila melanogaste*r by using various assays to screen mutant phenotypes. Mechanosensory functions of four TRPs - *nompC* in the TRPN subfamily, *painless* in the TRPA subfamily, and *nanchung* and *inactive* in the TRPV subfamily - have been determined, including roles in hearing, hygrosensing, and mechanical nociception (Eberl et al., 2000; Walker et al., 2000; Kim et al., 2003; Tracey et al., 2003; Gong et al., 2004; Liu et al., 2007) (Table 3.1).

I aimed to study the functions of several mechanosensory TRP channels described from *D. melanogaster* in the red flour beetle, *Tribolium castaneum.* This species diverged from the *D. melanogaster* lineage at least 300 million years ago (Grimaldi and Engel, 2005), but has several thermo-TRPs in common (Chapter 2) . *T. castaneum*, a stored-product pest, offers a unique opportunity to study the molecular mechanisms of mechanosensing because the target gene can be knocked down by systemic RNA interference (RNAi) at any target stages without any effects on previous stages (Tomoyasu and Denell, 2004). In *T. castaneum*, also offers a unique behavioral response to mechanical stimulation that allows a simple assay of mechanosensing. This behavior is known as tonic immobility; a beetle will become immobile for a period of time as a defensive response (Miyatake et al., 2004; Miyatake et al., 2008). Tonic immobility, an immobile status that serves as a defensive response, is a downstream response of mechanosensation in *T. castaneum* (Miyatake et al., 2004; Miyatake et al., 2008). This behavior is also called thanatosis, apparent death, playing dead, playing possum, and feigning death (Nakayama et al., 2012). As a response to mechanical stimuli, tonic immobility can be manually induced by touching the abdomen (Miyatake et al., 2004; Miyatake et al., 2008; Nakayama et al., 2012). The strength of tonic immobility is

heritable, and this behavior is strongly related to its genetic background (Prohammer and Wade, 1981; Miyatake et al., 2004). Therefore, a specific gene or group of genes likely affects the strength of this behavior. Based on previous mechanosensor studies in *D. melanogaster*, I identified candidate mechanosensing TRPs in *T. castaneum*. By phylogenetic analysis, I confirmed highly conserved one-to-one relationships between genes responsible for candidate TRPs in *D. melanogaster* and those in *T. castaneum*.

In addition to the candidate TRPs in the mechanosensation, I also identified and studied candidate downstream signal proteins that may be involved in tonic immobility. Insect cytokines (ICKs), secreted signaling proteins, are known for their diverse functions including hemocyte aggregation, morphogen (Tsuzuki et al., 2005), paralysis (Ha et al., 1999), growth retardation (Ohnishi et al., 1995), hemolymph aggregation (Nakatogawa et al., 2009), and stress responses (Kiyotake et al., 2014). Recently, a study with *T. castaneum* lines selected on the tonic immobility duration reported that the expression level of insect cytokine 1 (*ick1*) in the "short" tonic immobility line was increased when it compared to that of "long" tonic immobility line (Kiyotake et al., 2014). Based on amino acid sequence analysis (Fig. 3.2), two candidate ICK proteins *ick1* and *ick2*, in *T. castaneum* were evaluated for their possible function in mechanosensing.

To assess the mechanosensory functions of candidate TRP channels and ICK proteins in *T. castaneum*, I evaluated tonic immobility in beetles with different RNAi treatments of candidate TRP and ICK genes in *T. castaneum* female adults. In addition to the descriptions of obvious phenotypes in the lethality and the morphology in the RNAi-treated beetles, I also identified behavioral abnormalities.

Materials and Methods

Beetle cultures

I used virgin females of *T. castaneum* strain GA-1 (Haliscak and Beeman, 1983) for all experiments. Beetles were raised on whole wheat flour containing 5 % of brewer's yeast by weight. All beetles were kept in a growth chamber at 30 °C with ca. 40% relative humidity in 24:0 (L:D) lighting condition to make stable environment. The beetles were tested ten to 14 days after emerging to adults.

Phylogenetic analysis of TRPs and sequence alignment of insect cytokines

The amino acid sequences of seven *D. melanogaster* TRPs and eight *T. castaneum* TRPs in the TRPA, TRPN, and TRPV subfamilies (Matsuura et al., 2009) were retrieved from the NCBI database. The phylogenetic relationships were inferred for those 15 protein sequences. The amino acid sequences were aligned by the CLUSTAL W program (Thompson et al., 1994). The phylogenetic analyses were made by MEGA5 program (Kumar et al., 2008). The phylogenetic relationships were inferred with the UPGMA (unweighted pair group method with arithmetic mean) method with 1,000 bootstrap resampling (Fig. 3.1). The tree was built to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and were in the units of the number of amino acid substitutions per site. For TRPA5, I retrieved six TcTRPA5 orthologous sequences by blast searches in NCBI databases, and analyzed the phylogenetic relationship to check whether TRPA5 is separate from other TRPs. For ICKs analysis, 16 ICK amino acid sequences (Matsumoto et al., 2012) were retrieved

from the NCBI database. Five candidate ICKs in *T. castaneum* were captured by Prosite motif search (http://prosite.expasy.org). The amino acid sequences were aligned by the CLUSTAL W program (Thompson et al., 1994) (Fig. 3.2).

RNAi targeting candidate genes and quantitative-reverse transcription-PCR (RT-qPCR)

To synthesize DNA templates for double-stranded RNA (*dsRNA*) synthesis, I targeted the regions containing unique sequence of each cDNA. The primers used for *dsRNA* synthesis are in Table 2. The lengths of the cDNA for *dsRNA* synthesis were 707 bp for *dspainless*, 755 bp for *dspyrexia*, 480 bp for *dstrpA1*, 444 bp for *dsnanchung*, 512 bp for *dsinactive*, 475 bp for *dsnompC*, 511 bp for *dstrpA5*, 528 bp for *dswaterwitch*, 408 bp for *dsick1*, and 336 bp for *dsick2*. The PCR products amplified with a pair of primers containing T7 promoter sequences at the 5' ends were used for synthesizing *dsRNAs* by using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific). The *dsRNA* for *T. castaneum vermilion* was used as a negative control, for which the RNAi leads the loss of eye color, while other obvious phenotype has not been reported yet.

A total of 200 ng of *dsRNA* dissolved in 200 nl of injection buffer (0.1 mM sodium phosphate, 5 mM KCl, pH 7) with trace amount of food dye was injected into late stage of female pupae whose eye color was checked for age control. I made 13 treatment groups: three control groups for non-injected, buffer-injected, and *dsvermilion*-injected groups and ten experimental groups: *dspainless*, *dspyrexia*, *dstrpA1*, *dsnanchung*, *dsinactive*, *dsnompC*, *dstrpA5*, *dswaterwitch*, *dsick1*, and *dsick2*. All insects after *dsRNA* injection treatments were kept in a growth chamber at 30 °C with ca. 40% relative humidity in 24:0 (L:D) lighting condition. The survivorship and the phenotype after

injection were accessed in every other day. Three replications with ten individuals for each treatment were tested.

The efficiencies of the RNAi were accessed from the samples, pool of three to five female beetles of each treatment, after the assays measuring walking behavior and tonic immobility. The DNA-free RNA was obtained by Tri reagent (MRC, Cincinnati, OH) followed by Turbo DNase (Ambion). cDNA templates were synthesized from 500 ng of total RNA using ImProm-II reverse transcription system (Promega) with oligo (dT) 20-based protocol. Reverse transcription was followed by RT-qPCR using iQ SYBR Green Supermix (BioRad). Primers for the RT-qPCR assay were used for amplifying a 257 bp fragment for *painless*, a 532 bp for *pyrexia*, a 176 bp for *trpA1*, a 118 bp for *nanchung*, a 102 bp for *inactive*, a 110 bp for *nompC*, a 149 bp for *trpA5*, a 115 bp for *waterwitch*, a 362 bp for *ick1*, a 204 bp for *ick2*. A 182 bp amplicon encoding ribosomal protein S3 (*rps3*) served as a reference gene (Aikins et al., 2008) (Table 3.2). The specificities of amplicons were accessed by melting curves and electrophoreses on 1.0% agarose gels. Mean Ct values for the each gene were calculated from two technical repeats and used to calculate ΔΔCt values (Livak and Schmittgen, 2001). Fold-differences in the expression in each *dsRNA* injection treatment were calculated based on ΔΔCt values.

Behavioral analysis

The arena I used was made from a 3 mm thick aluminum plate with an oval shaped cutout (1.5 x 5 cm). The aluminum plate was placed on a filter paper and covered with a glass plate. One adult female beetle was placed in an oval cutout and video

recorded for 15 minutes, starting 30 second after it was introduced into the arena. All experiments were performed between 1 to 7PM. The walking speed of beetles in the arena was analyzed by EthoVision XT 7 (Noldus Information Technology, Wageningen, Netherlands). I calculated averages and standard errors of speed for three replications with five individuals per treatment. The significant differences of walking speed were analyzed by ANOVA and LSD multiple comparison tests at $p = 0.05$ level.

In addition, leg movement was observed and recorded to describe the slow movement of beetles, especially *dsnanchung* and *dsinactive* treated individuals. The angle between femur and tibia as they walked was also checked with video recording,.

Tonic immobility test

Each beetle was immobilized by gently turning it over and adhering its back to double sticky tape (3M Double coated urethane foam tape 4016 off-white, 3M, St. Paul, MN, USA). Tonic immobility was induced by touching the abdomen with a soft plastic stick, moving it from posterior to anterior of the ventral surface of the beetle. Every touch was counted as one trial. The leg movement of beetle was the major criterion for mobile and immobile status of beetle. The duration of immobility after each touch was measured. Consecutive stimulations after the beetle recovered were repeated up to ten times or until the immobile period was longer than 60 seconds. All experiments were performed between 1 to 7PM.

I defined the sensitivity of beetles to the mechanical stimulation by counting the percent of the trials that induce the tonic immobility. I set the threshold of the immobile duration as nine seconds for counting the frequency of significant tonic immobility,

because nine seconds was the 95 % significant level for the Poisson distribution of log-transformed tonic immobility durations from non-injected control in total 626 trials. The frequency of tonic immobility of 11 dsRNA treatments was analyzed with its means and standard errors of three biological replications with five individuals per replication.

Results

Phylogeny of TRPs and the genes containing ICK sequence motifs

Phylogenetic analysis for 15 amino acid sequences of *D. melanogaster* and *T. castaneum* TRPs suggested clear orthologous clusters of all three candidate subfamilies: TRPA, TRPV, and TRPN (Fig. 3.1). The one-to-one orthology was well conserved between the two species. Among five *T. castaneum* TRPA subfamilies, four had an orthologous relationship to each TRP in *D. melanogaster*: Painless, Pyrexia, TRPA1 and Waterwitch. With blastp search on the non-redundant (*nr*) database in NCBI, I confirmed six TRPA5 orthologous sequences in hymenopteran insects: *Apis mellifera* (XP_001122445.1)*, Camponotus floridanus* (EFN65541.1)*, Megachile rotundata* (XP_003700789.1)*, Bombus impatiens* (XP_003491223.1)*, Bombus terrestris* (XP_003395119.1), *and Solenopsis invicta* (BAN81931.1). All TRPA5 orthologous sequences were grouped together in the phylogenetic analysis with 15 TRPs in *D. melanogaster* and *T. castaneum*. In TRPN and TRPV subfamilies, the one-to-one orthologous relationship was also conserved between *D. melanogaster* and *T. castaneum*.

I used 16 ICKs sequences previously entered in NCBI in the alignment (Fig. 3.2). Five candidate ICKs in *T. castaneum* were captured by Prosite motif search (http://prosite.expasy.org) for the searches of the pattern C-x(2)-G-x(4, 6)-G-x(1, 2)-C-

[KR] (Prosite syntax rule) allowing one mismatch and occurring in the C-terminal end of the genes having smaller than total 300 amino acids for the putative translation. Among five putative TcICKs, TcICK1 was previously described (Matsumoto et al., 2012). I targeted two of the TcICKs, TcICK1 and TcICK2, to identify its function in tonic immobility

dsRNA efficiency

I confirmed the suppression of the target transcripts after *dsRNA* injection by Q-PCR and the eye color of *dsvermilion* injection. With comparison to non-injected control, each *dsRNA* treatments was successfully suppressed the expression of target genes – *vermilion*, *painless*, *pyrexia*, *trpA1*, *nanchung*, *inactive*, *nompC*, *trpA5*, *waterwitch*, *ick1*, and *ick2* – to 5 ~ 52 % (Fig. 3.S1). The lethality caused by injection was negligible with less than 10 % within two days after injection.

Effects on eclosion and morphology

With three replications of ten individuals per replication, *dsnompC* and *dstrpA5* injections resulted in 93 % of mortality in either treatment caused by failure in the eclosion. Therefore, the behavioral analysis and tonic immobility test was not conducted for these two *dsRNA* treatments. The arrest at eclosion occurred with the abdomen still remaining in old cuticle, while the head and thorax normally escaped from the old cuticular layer. The survivors of the *dsnompC* treatment (2 individuals in total 30) displayed defects in sclerotization of the elytra in posterior portion (Fig. 3.3).

A defect in the movement of hind legs, without obvious physical differences, was observed in all the individuals with *dsnanchung* and *dsinactive* treatments. The individuals glued on their back showed that the mobile angle between femur and tibia of *dsnanchung* and *dsinactive* treated individuals was ca. 45 to 180 ° (Fig. 3.4), while that of the control group was 0 to 180 °. In addition, less frequent strokes in the leg movements were observed in those individuals than that in control individuals. This defect was not found in other legs. The defect in the hind leg likely caused the slower walking speed we found in these beetles in the behavioral arena. The reduction in walking speed was significant; *dsnanchung* (0.15 ± 0.01 cm/sec., Mean ± S.E.) and *dsinactive* (0.16 ± 0.01) treatments walked more than 10 % slower than the non-injected control (0.18 ± 0.01) (Fig. 3.5) with $P < 0.0001$ in ANOVA ($F = 7.89$, $df = 10$). Unexpectedly, we observed a significant increase in walking speed with *dsvermilion* treatment, a negative control (0.25 ± 0.02 cm/sec., Mean ± S.E.).

Tonic immobility

The frequency of tonic immobility was significantly higher for *dsnanchung* (0.73 ± 0.07, Mean ± S.E.), *dsinactive* (0.75 ± 0.07), *dswaterwitch* (0.67 ± 0.07), and *dsick2* (0.64 ± 0.08) when compared to non-injected control (0.34 ± 0.09) with pairwise *t*-test ($p < 0.05$) (Fig. 3.6).

Discussion

Using the amino acid sequences of seven *D. melanogaster* TRPs and eight *T. castaneum* TRPs in the TRPA, TRPN, and TRPV subfamilies, I inferred phylogenetic

relationships for those 15 protein sequences. I assessed the phenotypes of *T. castaneum* after RNAi treatment of ten candidate genes, eight *trps* and two *icks*, to determine their involvement in mechanosensing. In addition, morphology and walking behavior of dsRNA-treated *T. castaneum* were evaluated. Based on my phylogenetic analysis of seven *D. melanogaster* and eight *T. castaneum* TRPs in TRPA, TRPN, and TRPV subfamilies, one-to-one orthologous relationships were found to be well conserved between the two species except for *TctrpA,* which was possibly lost in *D. melanogaster* (Fig. 3.1). The orthologue to *TctrpA5* was only found in six species of hymenopteran insects, but not in other taxa of insect for which genome sequences are available. All of these *TctrpA5* orthologues were grouped together with more than 90 % bootstrapping scores and separated from other TRPA subfamily members. Therefore, *trpA5* is likely a unique TRP channel that exists in Coleoptera and Hymenoptera .

Among the 13 treatment groups, I found two lethal phenotypes from *dsnompC* and *dstrpA5* that led to molting arrest in pupae (Fig. 3.3). NompC has been identified as a mechanosensor and proprioception in larval and adult locomotion in *D. melanogaster* (Cheng et al., 2010; Eberl et al., 2000; Walker et al., 2000). Mechanical proprioception feedback is likely required in emerging adults as part of the sequence of eclosion (Arakane et al., 2008). Therefore, NompC may be involved in sensing when the adult body is freed from old pupal cuticle and relays the behavioral sequence to post-eclosion behavior (Arakane et al., 2008). This interpretation is supported by observation of the two *dsnompC*-treated individuals in my study that successfully completed eclosion. In these individuals, the posterior part of elytra failed to normally sclerotize and they died within ten days after eclosion. The sclerotization of the elytra is believed to be associated with

the signaling molecules, bursicon, that also triggers post-eclosion behavior (Arakane et al., 2008). I suggest that the sensory input through NompC directly or indirectly interacts with the bursicon signal. For *trpA5*, I showed that *trpA5* is required to make successful emergence to adult in *T. castaneum* because *dstrpA5*-treated beetles failed to emerge adults. However, although most of members in TRPA subfamily in *D. melanogaster* are involved in thermosensing and mechanosensing, further research is needed to identify the function of *trpA5* in *T. castaneum*.

I measured the degree of tonic immobility to assess mechanosensory functions specifically through the abdominal mechanosensory input. I found that the frequency of tonic immobility was increased with four dsRNA treatments: *dsnanchung, dsinactive, dswaterwitch*, and *dsick2* (Fig. 3.6), while there was no case in which RNAi treatment resulted in less frequent or shorter tonic immobility compared to the control. Among the four treatments, *dsnanchung* and *dsinactive* also showed defects in the hind leg mobility, which was associated with sensitization for tonic immobility. *dsnanchung* and *dsinactive* in *T. castaneum* showed indistinguishable effects on walking speed and frequency of tonic immobility, reducing the former and increasing the latter (Fig 3.4, 3.5, and 3.6). In *D. melanogaste*r, *nanchung* and *inactive* together form a heteromultimeric channel that mediates hearing (Gong et al., 2004). Therefore, I suggest that in *T. castaneum* these two *trps* may similarly form a heteromultimeric channel as a functional unit for mechanosensing, especially proprioception between femur and tibia in the hind legs.

In this study, I showed that three of the candidate TRPs - *nanchung*, *inactive*, and *waterwitch* - are mechanosensors involved with tonic immobility. The candidate signalling protein *ick2* is likely mediating mechanosensing information transduction to

generate proper responses against mechanical stress in *T. castaneum* as a hormone or neurotransmitter. Based on this study, further study on mechanosensing mechanism in different life stages is recommended to understand the detailed mechanosensing mechanisms. In addition, the specific location of mechanosensors will be important to understand the mechanosensing mechanism.

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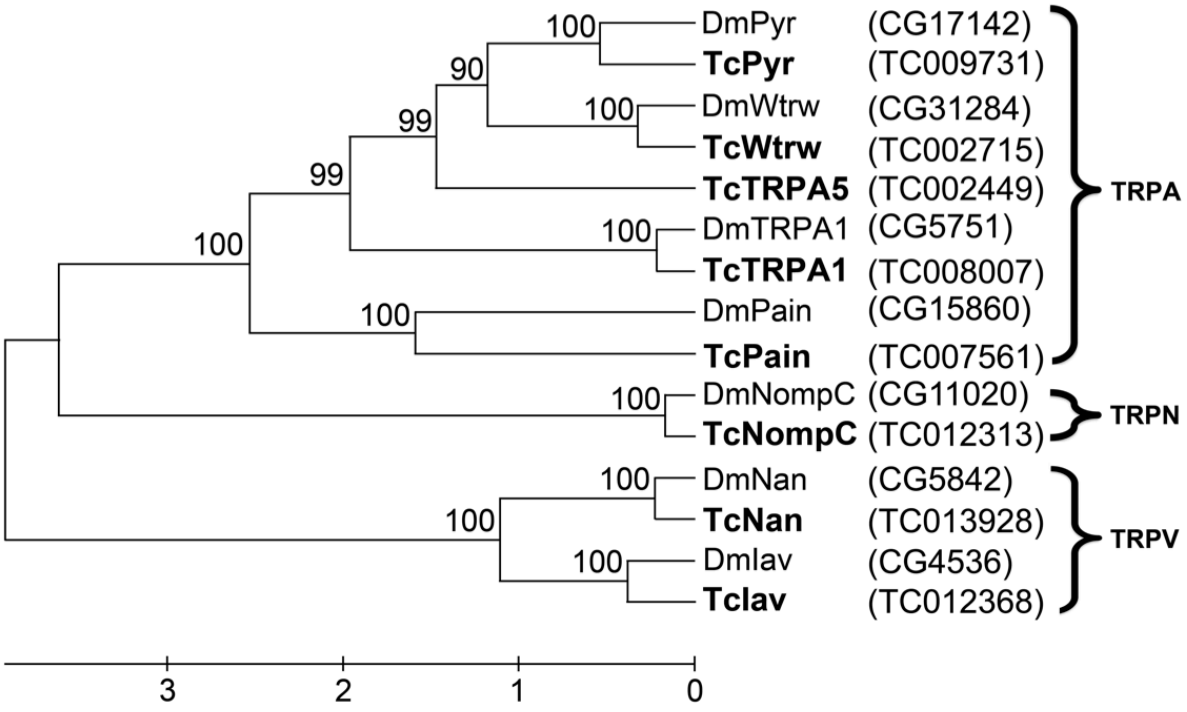

Fig. 3.1. Phylogenetic tree for 15 amino acid sequences from *D. melanogaster* and *T. castaneum* TRPs.

Seven D. melanogaster and eight T. castaneum TRPs belong to TRPA, TRPN, and TRPV subfamilies were aligned by the CLUSTAL W program. The phylogenetic tree was made by MEGA5 program with UPGMA method with 1,000 bootstrap resampling. The bootstrap scores were place on the branches.

Fig. 3.2. Sequence alignment of insect cytokines (ICKs).

The shaded three amino acid sequences are well conserved among 21 ICKs. The six potential insect cytokines (ICK) in *T. castaneum* are placed on the bottom and the two ICKs I tested are boxed.

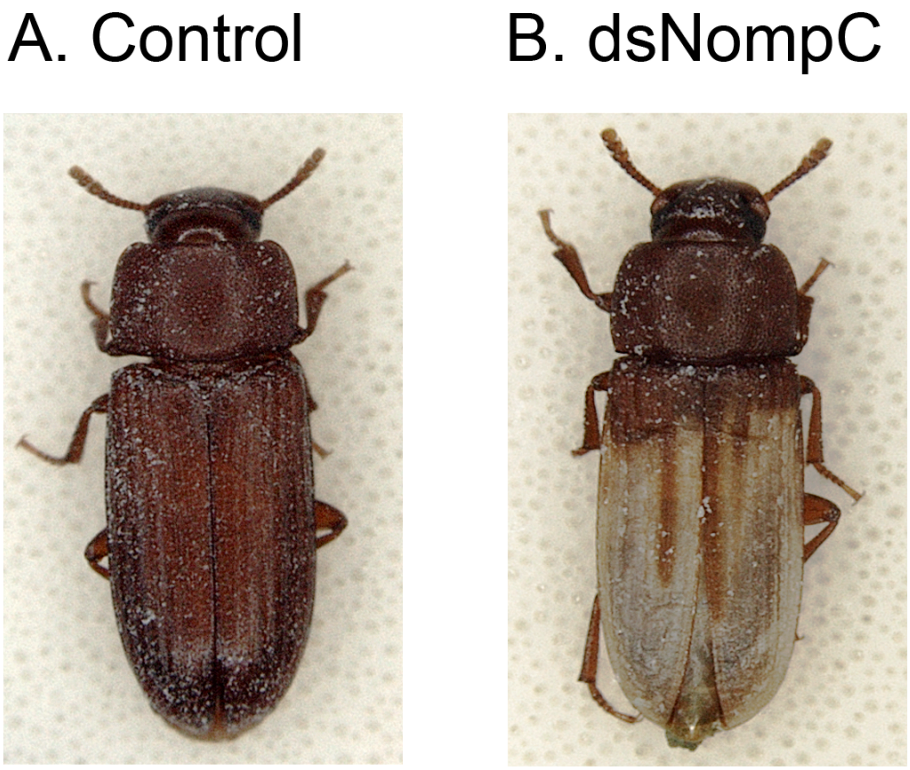

Fig. 3.3. Elytra development with *dsnompC* injection.

The color of elytra with *dsnompC* injection (B) is fainter than that of control individual (A). However, a borderline between normal sclerotized part and abnormal part was shown on the *dsnompC* injected beetle.

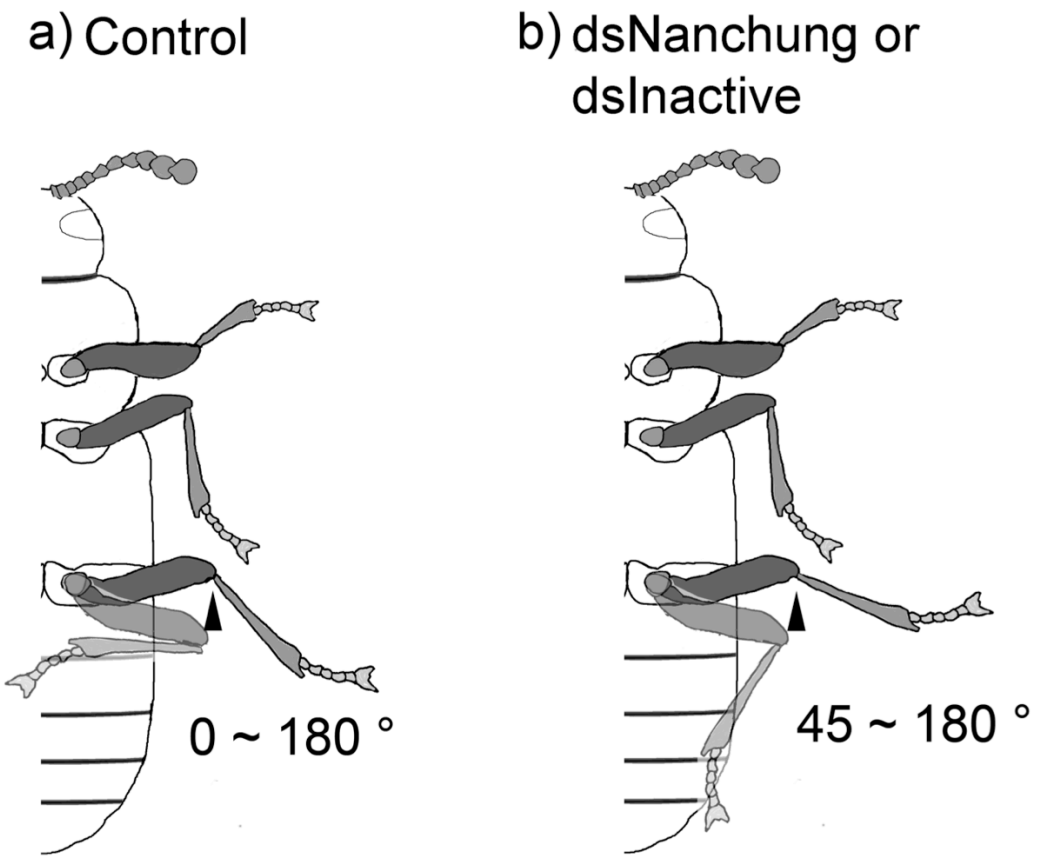

Fig. 3.4. Comparison of leg movement of *dsnanchung* and *dsinactive* treatments to that of control treatment.

The angle (wedge shape) between femur and tibia of hind leg is different. The angle was actively varied from 0 to 180 ° while the beetle walking for the control group. However, *dsRNA* treated individual made less movement of that angle between 45 and 180 ° while walking. However, the movement of other legs was not different.

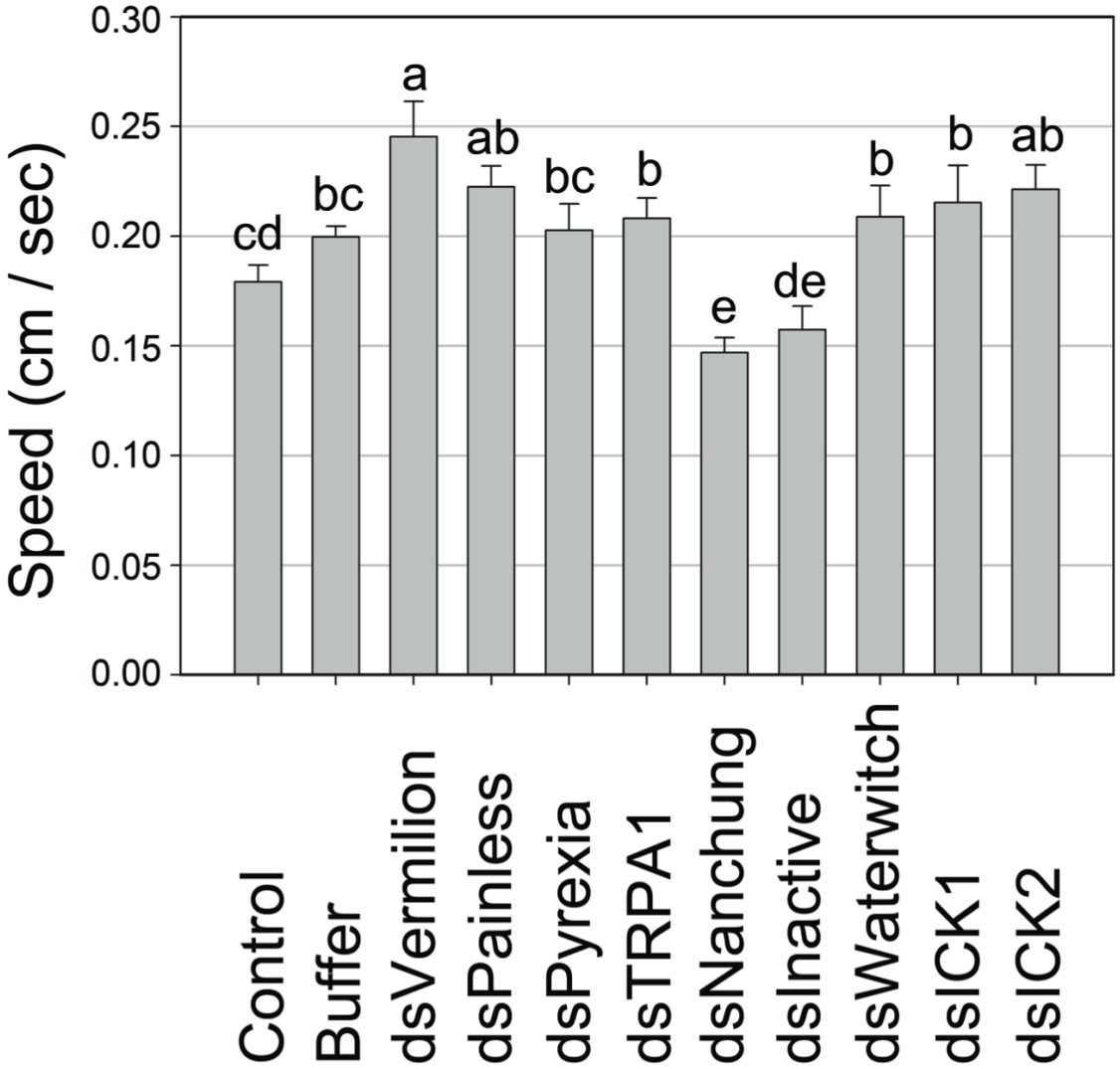

Fig. 3.5. Comparison of walking speed among 11 *dsRNA* treatments for three replications with five individuals per treatments.

Different letters above each column indicate significant difference detected by ANOVA ($F = 7.89$, $df = 10$, $P < 0.0001$) and LSD multiple comparison tests at P=0.05 level.

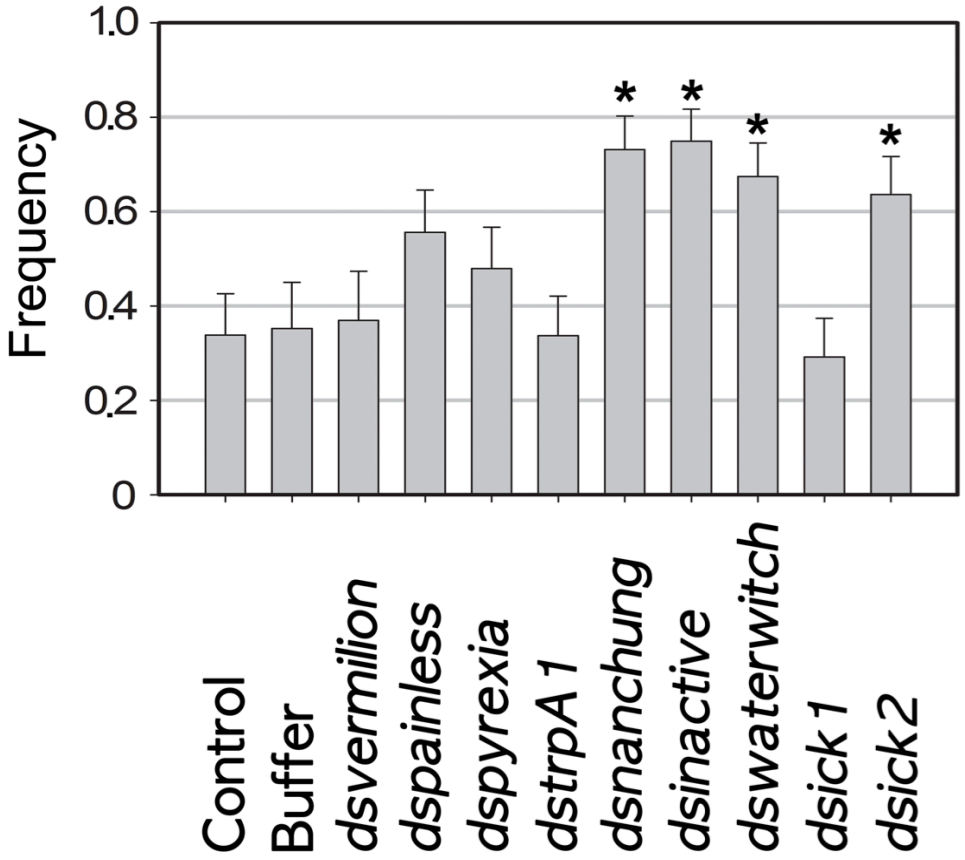

Fig. 3.6. Frequency of tonic immobility

The means and standard errors for the frequency of tonic immobility. Each *dsRNA* treatment was compared to the control treatment by pairwise t-test. * indicates $p < 0.05$. The sample size is five individuals with three replications.

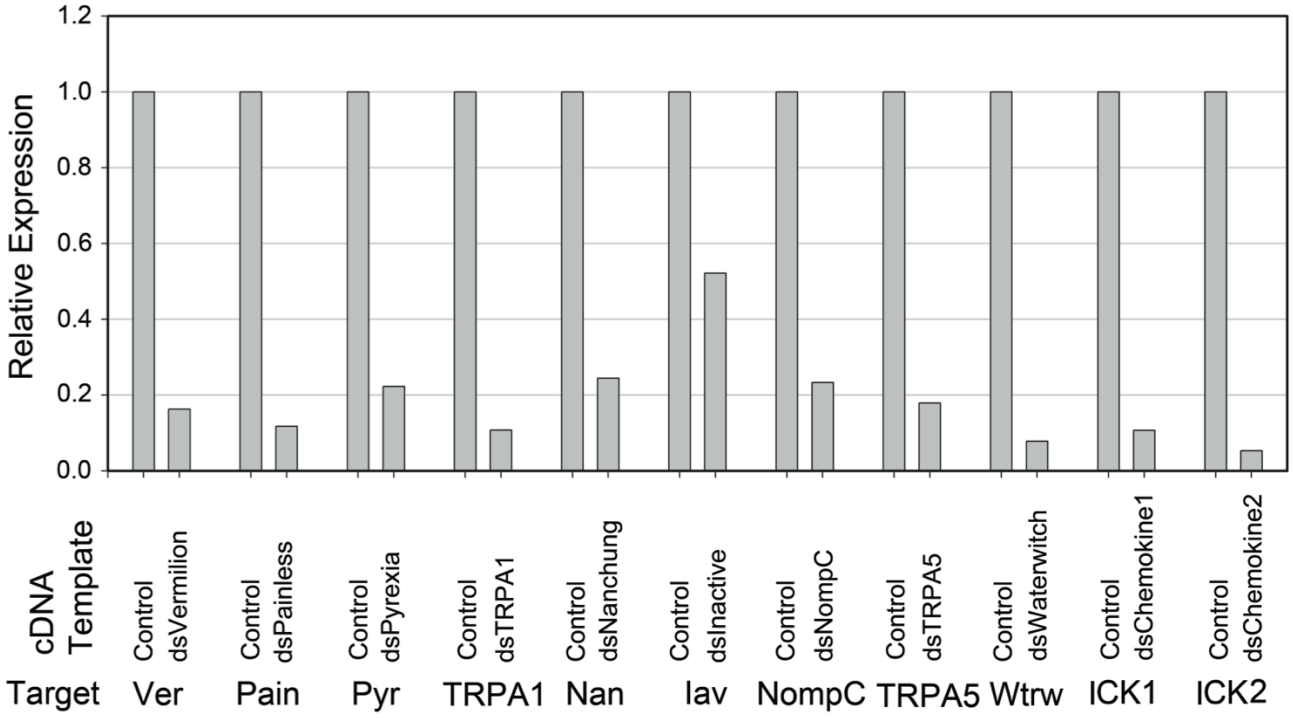

Fig. 3.S1. Effectiveness of *dsRNA* treatment.

Each bar represents two technical replications of RT-qPCR. Each pair of two bars for the same target gene showed *dsRNA* treatment was successful.

Table 3.1. Summary for functions of *trp* genes in *D. melanogaster* and *T. castaneum*

Subfamily	Gene		Annotation symbol	Mode of activation	Tested with	Functions identified in the study
TRPA	*painless*	*D. melanogaster*	CG15860	>38'C Mechanical stimuli	Mutant line	Thermal and mechanical nociception (Tracey et al., 2003)
		T. castaneum	TC007561	Heat	RNAi	Protect from heat stress with pre-exposure
	pyrexia	*D. melanogaster*	CG17142	>40'C	Mutant line	Keeping thermal preference and increasing thermal tolerance (Lee et al., 2005)
		T. castaneum	TC009731	Heat	RNAi	Protect from heat stress without pre-exposure
	waterwitch	*D. melanogaster*	CG31284	Moist air	Mutant line and RNAi	Hygrosensing (Liu et al., 2007)
		T. castaneum	TC002715	Mechanical stimuli	RNAi	Mechanoception in tonic immobility
	trpA1	*D. melanogaster*	CG5751	>27'C	RNAi	Thermosensing and thermotaxis (Rosenzweig et al., 2005)
		T. castaneum	TC008007	>39'C	RNAi	Thermosensing
	trpa5	*D. melanogaster*	N/A			
		T. castaneum	TC002449		RNAi	Arrested in eclosion
TRPN	*nompC*	*D. melanogaster*	CG11020	Tension	Mutant line Mutant line	Mechanosensory transduction (Walker et al., 2000) Hearing (Eberl et al., 2000)
		T. castaneum	TC012313	Mechanical stimuli	RNAi	Mechanical priprioception for molting Arrested in eclosion
TRPV	*nanchung*	*D. melanogaster*	CG5842	Hypoosmolarity Dry air	Mutant line Mutant line and RNAi	Hearing (Kim et al., 2003) Hygrosensing (Liu et al., 2007)
		T. castaneum	TC013928	Mechanical stimuli	RNAi	Mechanical priprioception Mechanoception in tonic immobility
	inactive	*D. melanogaster*	CG4536	Hypoosmolarity	Mutant line	Hearing (Gong et al., 2004)
		T. castaneum	TC012368	Mechanical stimuli	RNAi	Mechanical priprioception Mechanoception in tonic immobility

Abbreviation:RNAi, RNA interference

Table 3.2. The primers were used for *dsRNA* synthesis and RT-qPCR

	dsRNA	RT-qPCR
Painless	TAATACGACTCACTATAGGGCATCCCTCTTCACTACTCCC	GTATATTGACTCGCCCACTAGC
	TAATACGACTCACTATAGGGACCAACTCAAACGCTGCCAA	CCGTCTCAGTCCCATTCACA
Pyrexia	TAATACGACTCACTATAGGGCCGATAAACTCACCCCTCTG	CAGTTCGTGTTTCTCGCCTA
	TAATACGACTCACTATAGGGACACGGACGAGTTAGGAGA	TTATGCCCTCCATTTTCTCCCT
TRPA1	TAATACGACTCACTATAGGGTGCTCACAGTTTTACGCCGA	GTATCACCAAAGCCAACTGC
	TAATACGACTCACTATAGGGTGCCCCAAAAAATCGGAA	TCTACCGTGCGAAACCATTG
Vermilion	TAATACGACTCACTATAGGGGAGCAAATCGCCAAGTCGG	TTCTCACACAAAGCCCTGC
	TAATACGACTCACTATAGGGCCTGGGTTCGTCCCTGTAA	GCGAGTCTATGTCCATCAAAAG
Nanchung	TAATACGACTCACTATAGGGCTGCTGTGGCAACTTTATG	CGAGGATCCGTCAATGGTAAA
	TAATACGACTCACTATAGGGCTTATTTGGCCCGTTTCAATGT	GTCCAAGGAGTCATAACGAGAC
Inactive	TAATACGACTCACTATAGGGACCGGCGATATGTTGACTTT	CCTGGGTCTTGGTTTCTTCTC
	TAATACGACTCACTATAGGGAGCGCGTTTATCGTTACTTTG	AGTCAACATATCGCCGGTAATC
NompC	TAATACGACTCACTATAGGGCAGATGGTGGAGGTGTTGTTAG	CGAAGCTATGGCTACTGAACTG
	TAATACGACTCACTATAGGGCGATCAGTGGCCGTTAGTATTT	CGATGAGGACATCCAGAAACTC
TRPA5	TAATACGACTCACTATAGGGCGCCCAACACGACCATAAA	TTTGGGCCACGGTGTATTAG
	TAATACGACTCACTATAGGGCTCTATTCCCGTCACGCTTTC	CAGCCAGTTCATCCTCTCTATTC
Waterwitch	TAATACGACTCACTATAGGGCTACACATAGCCGCCTTGAA	GCGGACTAATCCGTTCGTTAT
	TAATACGACTCACTATAGGGCGAACCACTGCATTTCGATAAC	GTACTGCTTGACAGTGGGATAG
ICK1	TAATACGACTCACTATAGGGCTCAAGCTCAACGGTCATCA	CATCAGGATGCCGATTGTGA
	TAATACGACTCACTATAGGG CTTCCCTGCAATTCCCTTTT	CGAGAAGAATCCAAAACAAT
ICK2	TAATACGACTCACTATAGGG CGAAGGTCCTCTACGACCAG	GTGGACATCAGCAGAAGCAA
	TAATACGACTCACTATAGGG TGGGTGATTTTGCGATTACA	CTTCTTCCCGTCCTTTTTCC
RPS3		ACCGTCGTATTCGTGAATTGAC
		ACCTCGATACACCATAGCAAGC

Chapter 4 - Conclusion

Organisms need to detect environmental signals and sense their own body to survive in nature. Various sensors are specialized for accurate evaluation of internal and external stimuli through diverse modalities, including photoreception, chemoreception, mechanoreception, thermoreception, and proprioception. The specialized sensors for each stimulus are activated by changes in membrane channels when appropriate signals are applied. A group of membrane channel named transient receptor potential channels (TRPs) is known to function as photoreception, chemoreception, mechanoreception, and thermoreception. In my dissertation study, I focused on the function of some TRPs in *Tribolium castaneum* (Coleoptera: Tenebrionidae) as thermoreceptors in thermotaxis (behavior) and thermal acclimation (physiology), and some as mechanoreceptors in tonic immobility.

T. castaneum is a stored-product pest as well as a model genetic organism. Therefore, the genomic, physiological, and ecological information of this organism is broadly available. Moreover, *T. castaneum* is a good study organism because systemic RNA interference (RNAi) is able to easily knock down the expression of target genes at any target stage without effects on previous life stages.

From previous research, 14 TRPs were identified in *T. castaneum*. With a bioinformatics search, I found one new *trp* gene (TC007028, GenBank Accession number EFA02794.1) that is orthologous to the *Drosophila melanogaster trpGamma* gene from the genome database of *T. castaneum*. With phylogenetic analyses of 34 TRPs in *Homo sapiens*, *D. melanogaster*, and *T. castaneum*, I confirmed clear orthologous

clusters of all seven subfamilies of TRPs. The one-to-one orthologous relationship was generally conserved between *D. melanogaster* and *T. castaneum* except putative gene-losses in each TRPA and TRPC subfamilies of *D. melanogaster*.

With phylogenetic analyses and previous researches in *D. melanogaster*, I identified three candidate TRPs for high temperature sensing and eight candidate TRPs for mechanosensing in *T. castaneum*. To identify the function of candidate TRPs in thermosensation and mechanosensation in adult *T. castaneum*, I injected double stranded RNA (*dsRNA*) into late stage pupae and tested the treated individuals with 14 days after emerging adults.

In chapter 2, I identified the function of candidate TRPs as thermosensor in thermotaxis and thermal acclimation. To investigate thermotactic behavior, I developed an arena divided into two different temperature zones. Thermotactic behavior in *T. castaneum* was evaluated by measuring percentage of time spent in the control zone and relative speed using six different *dsRNA* treatments. These six dsRNA treatments were divided by three control groups (non-injected, buffer-injected, and *dsvermilion*-injected groups) and three experimental groups (*dspainless*-, *dspyrexia*- and *dstrpA1*-injected groups). With this behavioral analysis, I confirmed the function of TRPA1 as a thermosensor that activated at more than 39 °C in *T. castaneum*. The thermotactic behavior of *dstrpA1*-treated individuals was reduced when compared to control groups. However, other *dsRNA* treatments were not significantly different in thermotactic behavior. In *D. melanogaster*, TRPA1 was identified as the thermosensing channel for 24 ~ 29 °C. The difference in activation temperature between *D. melanogaster* and *T.*

castaneum may be the consequence of evolution for optimal temperature based on the different habitat or different life history.

The function of candidate TRPs in thermal acclimation was identified with a 10-minute pre-exposure to 42 °C. First, I showed that acclimation with 10-minute pre-exposure to 42 °C increased the time required for heat-induced knockout under subsequent high temperature stress (one minute at 52 °C). This type of short period thermal acclimation is likely mediated by sensory mechanisms. With *dsRNA* treatment, I identified the function of TRPA1 as a thermosensor in thermal acclimation with the time required for heat-induced knockout. High temperature sensing through TRPA1 increased the resistance to heat-induced knockout in *T. castaneum* without any effect on long-term survivorship. Without thermal acclimation, *pyrexia* RNAi treatment reduced the maximum recovery rate after heat treatment. RNAi treatment of *painless* lowered ability in thermal acclimation, which is shown by reduced long-term survivorship with 10-minute pre-exposure to 42 °C.

In chapter 3, the mechanosensory function of eight candidate TRPs and two candidate insect cytokines (ICKs) was investigated using tonic immobility. I also evaluated the effects of *dsRNA* treatments on these candidates on obvious phenotypes in morphology, lethality, and behavioral abnormality in *T. castaneum*. With both *dsnompC* and *dstrpA5* treatments, I observed 93 % of mortality caused by failure in eclosion. Given the high mortality caused by pupal arrestment, the function of *nompC* and *trpA5* is likely mechanical proprioception for molting behavior. Those individuals in the *dsnompC* treatment that completed eclosion failed to normal sclerotize the posterior part of elytra and died within ten days after eclosion. This failure suggests the involvement of sensory

input from *nompC* in the normal sclerotization of elytra. With behavioral analysis, the reduced walking speed in *dsnanchung* and *dsinactive* treated beetles was observed to e associated with defects in the movement of hind legs. The mobile angle between femur and tibia was reduced with these two *dsRNA* treatments when compared to other treatments. The indistinguishable effects of *nanchung* and *inactive* were also identified in *D. melanogaster*, so these two TRPs are likely forming a heteromultimeric channel as a functional unit for mechanical proprioception between femur and tibia in the hind legs.

Tonic immobility, an obvious behavioral response to mechanical stimuli, was induced by touching the abdomen from a posterior to anterior direction on the ventral surface of beetles with a soft plastic stick. Among 13 *dsRNA* treatments - three controls, eight TRPs and two ICKs –*dsnanchung*, *dsinactive*, *dswaterwitch*, and *dsick2* increased the frequency of tonic immobility. Taking all of these results combined, I concluded the three TRPs are likely mechanosensors and *ick2*, a secreted signal protein, may mediate the mechanical stimulus.

In my dissertation, I identified the function of candidate TRPs as thermosensors in thermotaxis and thermal acclimation, and as mechanosensors in tonic immobility. In addition, I showed that short time pre-exposure to lethal temperature was enough to induce thermal acclimation in *T. castaneum*, and mechanical proprioception was required for normal sclerotization of adult cuticle in *T. castaneum*. With accurate evaluation of environmental stimuli, organisms can initiate the behavioral and physiological responses to maintain the organismal homeostasis and the fundamental biochemistry of cellular metabolism, and to escape from life threatening event such as predation. Otherwise, organisms should encounter disadvantageous or critical status including knockout,

predation, and death. It may be possible to develop alternative pest management strategies for *T. castaneum* and related pest by disturbing sensing mechanisms that I have identified. Moreover, this information will serve as a foundation to genetic, physiological, and ecological studies for thermosensing and mechanosensing in *T. castaneum*.