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GENE RESPONSE TO STRESS IN THE ASIAN CITRUS PSYLLID (HEMIPTERA: PSYLLIDAE)

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri,* is a vector of the phloem-inhabiting bacterium *Candidatus* Liberibacter asiaticus, a pathogen associated with the economically important citrus disease known as huanglongbing. Knowledge of the molecular genetics of *D. citri* and other insects provides insights into the basic biology of insects. For example, insects can be subjected to stressful conditions and then screened to determine if the conditions promote specific genetic responses. Such information, by identifying critical genetic responses linked to survival, can then be used in the development of genetic tools and in novel management strategies aimed at reducing psyllids populations. In this study, transcriptional responses of *D. citri* adults against 3 stress factors were investigated: physical wounding, heat stress, and exposure to low doses of the insecticide imidacloprid. No measureable transcriptional activity was observed for genes (*cyp, gst, CuZn-SOD, hsc70, or hsp90*), which, in other insects, have been shown to respond to either physical wounding, heat stress, or exposure to insecticides. However, increased transcriptional activity of a heat-shock gene, *hsp70*, was found in adult psyllids exposed to 42°C, although 6 h of exposure to this temperature was lethal to psyllids. These results suggest that *hsp70* may play a role in response to heat stress of *D. citri*. Summer temperatures can exceed 37°C in Florida, Texas and California areas where the psyllids now occurs. Natural temperature fluctuations and gradual increases provide enough time for psyllids to acclimate to hot summer temperatures. We propose that the development of a method to disrupt gene expression, such as *hsp70*, may be applicable for future strategies to suppress psyllids populations.

Key Words: Asian citrus psyllid, *Diaphorina citri*, gene expression, HLB, heat shock proteins, imidacloprid, population suppression

RESUMEN

El sílido asiático de los cítricos, *Diaphorina citri,* es un vector de la bacteria, *Candidatus* Liberibacter asiaticus que habita el floema y que es un patógeno asociado con una enfermedad de importancia económica conocida como huanglongbing. El conocimiento de la genética molecular de *D. citri* y otros insectos provee una perspectiva a la biología básica de insectos. Por ejemplo, los insectos pueden ser sometidos a condiciones de estrés y luego evaluados para determinar si estas condiciones promueven una respuesta genética específica. Dicha información, que identifica respuestas genéticas críticas que estan relacionadas a la sobrevivencia, puede ser usada en el desarrollo de herramientas genéticas y estrategias novedosas de manejo dirigidas a reducir poblaciones de sílidos. Investigamos las respuestas transcripcionales de los adultos de *D. citri* contra 3 factores de estrés: heridas físicas, estrés de calor, y exposición a las dosis bajas del insecticida imidacloprid. No actividad transcripcional medible fue observada en los genes (*cyp, gst, CuZn-SOD, hsc70,* o *hsp90*) que han muestrado respuestas a heridas físicas, estrés de calor, o exposición a las dosis bajas de insecticidas en otros insectos. Sin embargo, un aumento en la actividad transcripcional del gene al choque de calor, *hsp70*, fue encontrado en adultos de sílidos expuestos a 42°C, aunque la exposición de 6 horas a esta temperatura fue letal para los sílidos. Estos resultados indican que que el gene *hsp70* puede jugar un papel en la respuesta de *D. citri* al estrés de calor. Las temperaturas de verano pueden ser mas de 37°C en areas de la Florida, Texas, y California donde el sílido ahora ocurre. Fluctaciones naturales y aumentos graduales de temperatura provee suficiente tiempo para que los sílidos se aclimaten a las temperaturas calorosas de verano. Proponemos que el desarrollo de un método para perturbar la expressión genética, como en el gene *hsp70*, puede ser aplicable a estrategias para suprimir poblaciones de sílidos en un futuro.

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is a serious pest of citrus because it vectors *Candidatus* Liberibacter asiaticus, a bacterium associated with the severe citrus tree disease huanglongbing (HLB) (Bové 2006). HLB threatens the viability and production of citrus worldwide (Halbert & Manjunath 2004).

Previously, we constructed 2 cDNA libraries from field-collected adult *D. citri* to advance the

understanding of psyllids biology (Hunter et al. 2009; Marutani-Hert et al. 2009). With no reports about psyllids gene expression thus far, this study aimed at investigating some of the known immunity related transcripts identified from mining the available cDNA datasets. Preliminary results, partially published by Hunter et al. (2009), showed several homologies to known stress-response gene products such as heat shock proteins (HSP), detoxification enzymes, cytochrome P450s (CYP), glutathione S-transferase (GST), and copper zinc-superoxide dismutase (CuZn-SOD). HSP transcriptional expressions have been correlated with stress tolerance and gradients of environmental stress (Feder & Hofmann 1999). CYP450s are a superfamily of enzymes that are involved in the biosynthesis of many biologically important compounds for cell metabolism and detoxification (Feyereisen 1999). GST is known to be important for xenobiotic stress adaption resulting in resistance against environmental toxins and mutagens (Hayes & Pulford 1995). CuZn-SOD is a eukaryotic cytoplasmic enzyme that performs a protective function in the cell by scavenging superoxide radicals and dismutating them to hydrogen peroxide (McCord & Fridovich 1969).

Understanding the genetic basis of *D. citri* responses to stresses not only increases the basic understanding of insect immunity, but also identifies genetic targets for future development of insect genetic-disruption strategies aimed at pest suppression. The concept of transcriptional analyses and application to gene regulation by RNA interference (RNAi) techniques is widely considered and applied in plant protection research and is now rapidly being considered as an emerging strategy of insect management (Eamens et al. 2008). For example, ingestion of double strand RNAs supplied in artificial diet triggers RNAi in several coleopteran species (e.g., western corn rootworm, *Diabrotica virgifera virgifera* LeConte) (Baum et al. 2007). However, prior to conducting larger trials these genetic targets need to be screened for activity and function. Therefore, we investigated the transcriptional response of selected genes to stress induced by heat, pesticide exposure, and physical wounding.

Materials and Methods

Identification of Target Genes

Previously, a set of the 4,595 bp ESTs from adult *D. citri* was established (Hunter et al. 2009). Putative sequence identities were determined based on BLAST similarity searches (BLASTX and BLASTn). Transcripts linked to detoxification or immune responses in other insects that had E-value less than 1^{-20} were further analyzed. In this study, *cyp, gst, CuZn-SOD, hsc70, hsp70, and hsp90* homologues were selected to analyze transcriptional activity against stresses.

Source of Psyllids

Psyllid colonies were started from field collected adults (Fort Pierce, FL). The original psyllids were collected in 2004 and have since been maintained on the host plant cv. 'Madam vinous' sweet orange (*Citrus sinensis* (L.) Osbeck) in an insectary at ~25°C. Adults used for experiments were collected from the insectary reared colonies and held at 23°C in the laboratory in a cage with plants for 1 week prior to treatment applications.

Immune Challenge of Psyllids

For each treatment, a group of 50 psyllids was transferred to a 4-leaf plant branch in a vial with water in a small cage (6 × 6 × 10 cm). For control, psyllids on a plant branch were incubated at 23°C for 1, 3, 6, or 24 h. Heat shock responses were investigated by incubating psyllid–infested branches at 42°C for 1, 3, or 6 h. Responses to physical wounds to the body were investigated by wounding the abdomen with the point of a sterilized insect pin (size 2), after which the psyllids were incubated on a plant branch for 1, 3, 6, or 24 h at 23°C. Responses to a pesticide treatment were evaluated by maintaining 4 uninfested branches at 23°C in separate cages in vials with a solution containing 2.64 µL/L of imidacloprid (Admire® Pro Systemic Protectant, Bayer CropScience, Triangle Park, NC); after 3 d, 50 psyllids were transferred to each pesticide treated branch and maintained for 1, 3, 6, or 24 h at 23°C.

Isolation of RNA and Quantitative Real-time RT-PCR

RNA was extracted from psyllids (20 to 50) surviving treatments with RNA Aqueous®-Micro (Ambion, Austin, TX), following the manufacturer's instructions. This kit includes a DNA-free system that was utilized during extraction. RNA concentrations were determined with the NanoDrop™ ND-1000 spectrophotometer (NanoDrop™ Technologies, Wilmington, DE, USA). To confirm the absence of contaminating DNA, conventional PCR was conducted with Platinum PCR® SuperMix (Invitrogen, Carlsbad, CA, USA) and 200 ng of RNA as template. One-step quantitative real-time reverse transcriptase (RT) PCR was performed with the Rotor-Gene™ 3000 Real-time rotary analyzer (Corbett Life Science, Sydney, Australia) with SuperScript™ III Platinum® SYBR® Green OneStep qRT-PCR kit (Invitrogen, Carlsbad, CA). Each reaction was carried out in 15-µL volume containing 5 pmol of forward and reverse primers and 200 ng of RNA template. Primer sequences were determined with Primer3 (Rozen & Skaletsky 2000) and are listed in Table 1. For all genes except *hsc70* and *hsp90*, amplification cycling conditions were 50°C for 30 min, 95°C for 15 min, then 40 cycles of 95°C for 30

TABLE 1. SELECTED TRANSCRIPTS IN *DIAPHORINA CITRI* ANALYZED BY QUANTITATIVE REAL TIME RT-PCR.

Sequence	Accession #	Homology	Forward primer	Reverse primer
WHDc0267	DQ675542	cytochrome P450 (pea aphid) (79%) CYP4G25 (silkmoth) (75%)	5'-AGGTCTTGTGTGGGACGTAA-3'	5'-AACGGTTAGGCCACAGTTTG-3'
CMDc002_C03	AB475104	heat shock 70 protein (Gmelin, olive fruit fly) (61%)	5'-CGCTCAAATGGTTGGATAAC-3'	5'-AGCACCTCCATGCATCTTAC-3'
WHc0146	DQ675540	heat shock cognate 70 protein (fire bug) (89%) heat shock cognate 70 protein (whitefly) (87%)	5'-ACCTACTCTGACAACCAACC-3'	5'-ACCTACTCTGACAACCAACC-3'
WHDc019_A04	AB475103	heat shock 90 protein (honey bee) (86%) heat shock 90 protein (black-legged tick) (84%)	5'-GTTGAGAGAGTGAAGAAGCG-3'	5'-CCAGGATATCCTTCATCACC-3'
WHDc0680	DQ673416	ribosomal protein 18S	5'-CATTCTCCGTGTGATGTCCA-3'	5'-TTCACTGCATTCACCAGCTC-3'
WHDc055_A03	AB475105	copper zinc superoxide dismutase (pink hibiscus mealybug) (74%) copper zinc superoxide dismutase (pea aphid) (74%)	5'-CACGAATTTGGAGACAACACA-3'	5'-TGCCTAGGTCACCAACATGA-3'
WHDc1033	DQ675549	glutathione S-transferase-like protein (fruit fly) (55%) glutathione S-transferase-like protein (house fly) (51%)	5'-CCACATTGGAGGATGGAGAC-3'	5'-AGTTAGAGCCCTGGTCGACC-3'

s, 60°C for 30 s and 72°C for 30 s. To analyze *hsc70* and *hsp90*, 54°C was used as annealing temperature. PCR efficiencies of all genes (calculated dynamically with the Rotor-Gene™ 3000 Real-time rotary analyzer) were similar. Expression values were calculated by $2^{-\Delta\Delta Ct}$ methods (Livak & Schmittgen 2001). The data were presented as the fold change in target gene expression normalized to the 18s rRNA. In the present study, increased mRNA expression was defined as N-fold ≥2.0, "normal" expression was an N-fold ranging from 0.5001 to 1.9999, and decreased mRNA expression was N-fold ≤0.5. ANOVA was used to evaluate differences between treatments. A P value <0.01 was considered to be significant. Validations were checked with qBaseplus (Biogazelle, Zulte, Belgium) (Hellemans et al. 2007). The qBaseplus software determines relative gene expression levels by normalization to both PCR amplification efficiency and 18s rRNA abundance. All real-time quantitative RT-PCR assays were conducted in 3 technical replicates, and 3 repetitions of the experiment were performed with independently prepared RNA templates.

Results and Discussion

From the 2 available cDNA libraries of *D. citri* (Hunter et al. 2009), 6 clones were identified that contained sequence homologs to genes involved in stress responses (Table 1). Clone *WHDc0267* (accession number DQ675542) was 192 bp long and its predicted protein sequence had 79% homology with a partial predicted cytochrome P450 in *Acyrthosiphon pisum* (Harris) (pea aphid) (accession number XP_001944205) and 75% homology with the cytochrome P450 CYP4G25 of *Antheraea yamamai* (Guerin-Meneville) (silkmoth) (accession number BAD81026). The clone *CMDc002_C03* (accession number AB475104) was 179 bp long with a predicted protein sequence that had 61% homology to a partial sequence of a heat shock 70 protein in *Bactrocera oleae* or *Dacus oleae* (Gmelin) (olive fruit fly) (accession number CAI44197) (Drosopoulou et al. 2009). Clone *WHDc0146* (accession number DQ675540) was 550 bp long with 89% and 87% homology to a heat shock cognate 70 protein in *Pyrrhocoris apterus* (L) (fire bug) (accession number ACJ12782) (Kostal & Tollarova-Borovanska 2009) and a partial sequence of a heat shock cognate 70 in *Bemisia tabaci* (Gennadius) (whitefly) (accession number AAZ17399), respectively. Clone *WHDc019_A04* (accession number AB475103) was 705 bp long with 86% protein homology to a partial sequence of a putative heat shock 90 protein found in *Apis mellifera* L. (honey bee) (accession number XP_395168) and 84% homology to a HSP90 in *Ixodes scapularis* Say (black-legged tick) accession number EEC18473). Clone *WHDc1033* (accession number DQ675549) was 558 bp long with a predicted protein sequence homologous to partial sequences of glutathione S-transferase proteins found in *Drosophila ananassae* (Dobzhansky and Dreyfus) (fruit fly) (accession number XP_001959208) (55% homology) and *Musca domestica* L. (house fly) (accession number AAD54938) (51% homology). Clone *WHDc055_A03* (accession number AB475105) was 504 bp long with a predicted protein sequence homologous to partial sequences of copper-zinc superoxide dismutases found in *Maconellicoccus hirsutus* Green (pink hibiscus mealybug) (accession number ABM55632) (74% homology) and *Acyrthosiphon pisum* (pea aphid) (accession number XP_001951810) (74% homology).

Imidacloprid is one of the most common systemic insecticides currently used to reduce *D. citri* infestations in citrus (Rogers 2008). Psyllids were susceptible to imidacloprid. Survival in control treatments was 100%. In contrast, when psyllids were maintained on leaves treated with imidacloprid (2.64 µL/L), 96.9% ± 2.3% of psyllids were alive after 1 h; 92.4% ± 6.3% were alive after 3 h; and 49.8% ± 14.5% were alive after 24 h. In psyllids surviving this treatment, none of the 6 examined gene transcripts were determined to be altered (Fig. 1A). In contrast, in a DDT resistant *Anopheles gambiae* strain a resistance-related gene of the GST family, *aggst3-2*, was transcribed 5-fold more than in a susceptible strain (Ranson et al. 2001). The *Cyp6* group of genes has been reported to produce enzymes that are capable of metabolizing imidacloprid (such as *Cyp6g1* of *Drosophila melanogaster* Meig) (Joussen et al. 2008). Clone *WHDc0267* is from the *Cyp4* group and has predicted protein homology to CYP4G25 of *Antheraea yamamai* and CYP4C1 in *Blaberus discoidalis* Serville, which produce enzymes associated with fatty acid metabolism (Bradfield et al. 1991) and to CYP4C7 from *Diploptera punctata* (Eschscholtz), which has been shown to be active in juvenile hormone metabolism (Sutherland et al. 1998). Thus clone *WHDc0267* may play a major role in endogenous compound metabolism, but did not appear to assist in detoxification of ingested imidacloprid. Among adults subjected to heat treatments at 42°C, 2.6% ± 1.5% of psyllids died during a 1-h exposure period, 54.9% ± 7.5% died during a 3-h period, and 100% died during a 6-h period. The results showed that extremely hot temperatures can be lethal to psyllids. One hour and 3 h of heat shock treatments induced *hsp70* transcription significantly ($P < 0.01$, Fig. 1B). The *hsp* gene family consists of stress inducible (*hsp*) and constitutively expressed (*hsc*) forms that differ in their structure and levels of expression under changing environmental conditions (Sonoda et al. 2007). In *Mamestra brassicae*, while *hsc70* transcripts are expressed constitutively, the ex-

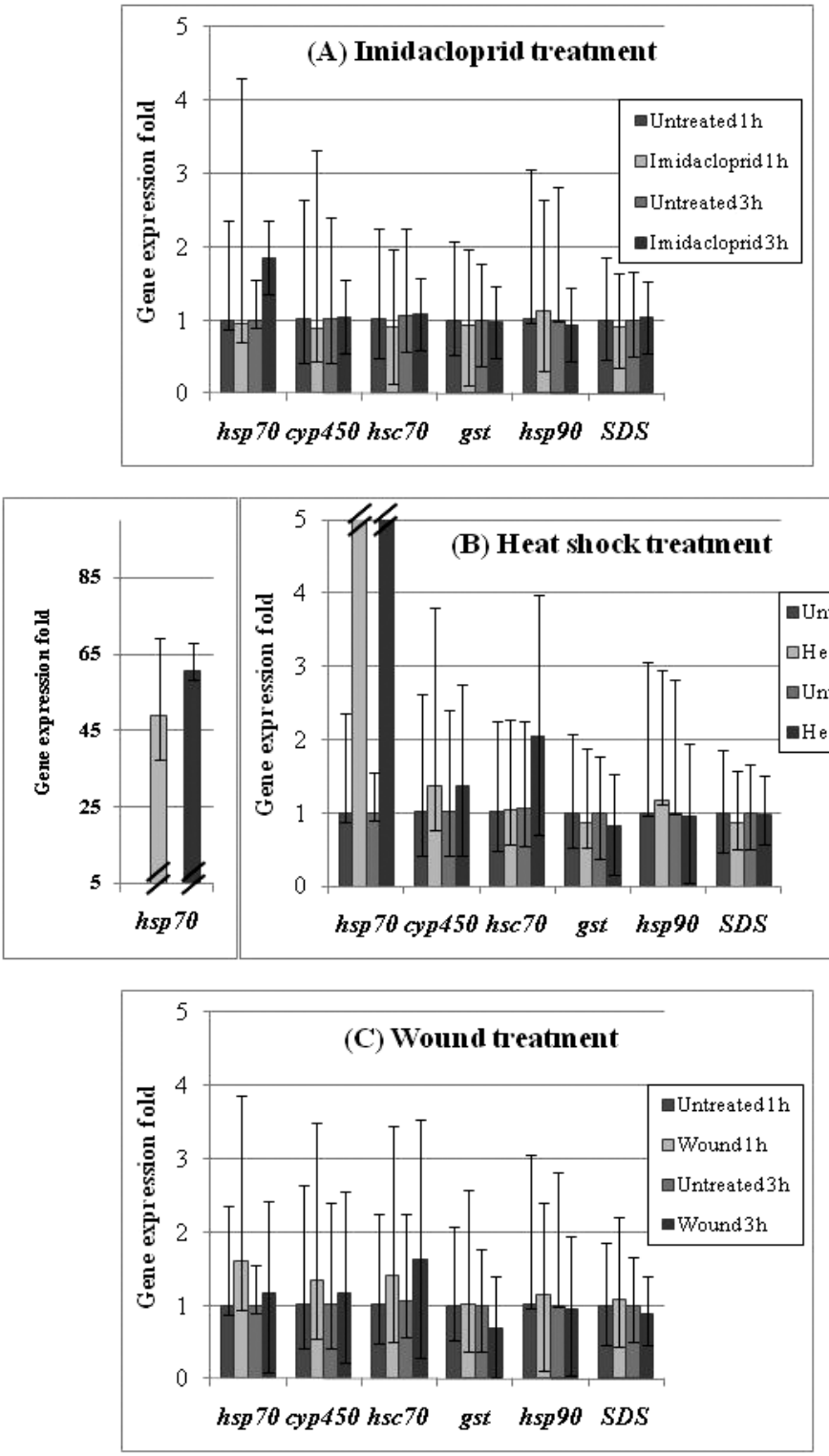

Fig. 1 Quantitative real-time RT-PCR analysis of transcriptional levels of six transcripts in adult *Diaphorina citri* exposed to (A) insecticide (imidacloprid), (B) heat (42°C), or (C) wounding. Lines on bars represent the range of transcriptional N-fold change.

pression of *hsp70* and *hsp90* changed in response to heat shock treatment (Sonoda et al. 2007). In *Pyrrhocoris apterus*, the expression of *hsp70* was up-regulated 3243-fold in response to heat; *hsc70* was up-regulated 2.6-fold (Kostal & Tollarova-Borovanska 2009). Our results showed that while *hsp70* transcription in *D. citri* was induced by heat shock treatment, transcript expression of *hsc70, hsp90, cyp450*, *gst*, or *CuZn-SOD* did not change in response to heat stress at either 1-h or 3-h exposures.

Among adults subjected to physical wounding, all psyllids survived 24 h post wounding (Fig. 1C). Heat shock genes produce proteins that aid survival of organisms to a variety of stresses, such as exposure to xenobiotics, heavy metals, metabolic poisons and temperature extremes (Feder & Hofmann 1999). For example, expression of the *hsp68* gene family was reported to be induced in *Tribolium castaneum* in response to both wounding and heat shock (Altincicek et al. 2008). However, in *D. citri*, none of the examined genes including *hsp70* and *hsp90* were shown to be induced by wounding.

In this study, we investigated transcriptional expression of 6 potentially stress-related *D. citri* genes in response to heat, wounding, and insecticide sources of stress. While expression of the *hsp70* transcript was not induced by imidacloprid exposure or wounding, it was induced by a heat treatment. In *D. melanogaster*, inducible thermotolerance is severely reduced in the *hsp70* null strain and increases linearly with *hsp70* copy number (Bettencourt et al. 2008). It is possible that in psyllids *hsp70* may have an important role as temperatures increase. The reduced survival rates showed that psyllids are sensitive to heat stress. To what extremes *D. citri* acclimates to hot weather is not yet known, and further experiments examining *hsp70* gene responses to rising temperatures are needed to determine whether disruption of *hsp70* gene expression may provide a potential future tool in psyllid population management.

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