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Cytochrome P450 gene, *CYP4G51*, modulates hydrocarbon production in the pea aphid, *Acyrthosiphon pisum*

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ABSTRACT

Terrestrial insects deposit a layer of hydrocarbons (HCs) as waterproofing agents on their epicuticle. The insect-specific CYP4G genes, subfamily members of P450, have been found in all insects with sequenced genomes to date. They are critical for HC biosynthesis in *Drosophila*; however, their functional roles in other insects including the piercing-sucking hemipterous aphids remain unknown. In this study, we presented the molecular characterization and a functional study of the *CYP4G51* gene in the pea aphid, *Acyrthosiphon pisum* (Harris). *CYP4G51* transcript was detectable across the whole life cycle of *A. pisum*, and was prominently expressed in the aphid head and abdominal cuticle. Up-regulation of *CYP4G51* under desiccation stress was more significant in the third instar nymphs compared with the adults. Also, up-regulation of *CYP4G51* was observed when the aphids fed on an artificial diet compared with those fed on the broad bean plant, and was positively correlated with a high level of cuticular HCs (CHCs). RNAi knockdown of *CYP4G51* significantly reduced its expression and caused reductions in both internal and external HCs. A deficiency in CHCs resulted in aphids being more susceptible to desiccation, with increased mortality under desiccation stress. The current results confirm that *CYP4G51* modulates HC biosynthesis to protect aphids from desiccation. Moreover, our data also indicate that saturated and straight-chain HCs play a major role in cuticular waterproofing in the pea aphid. *A. pisum CYP4G51* could be considered as a novel RNAi target in the field of insect pest management.

1. Introduction

Insects are the most diverse and the largest group in the animal kingdom. They are relatively small-sized and particularly vulnerable to desiccation due to their large surface area to volume ratio. The success of terrestrial insects is largely due to their ability to retain water. In the course of their evolution, insects have evolved a way of depositing a thin layer of long-chain hydrocarbons (HCs) on the epicuticle to reduce evaporative water loss (Gibbs, 1998). Cuticular HCs (CHCs) are produced by some discrete clusters of specialized cells termed oenocytes, which are originally derived from the ectoderm (Makki et al., 2014; Martins and Ramalho-Ortigão, 2012; Schal et al., 1998b) and are transported via lipophorin to reach the epicuticle (Blomquist and Bagnères, 2010; Schal et al., 1998b). Insect CHCs are usually highly diverse (Blomquist et al., 1987; Lockey, 1988), and are composed of a blend of C_{21}–C_{37} alkanes, alkenes and their methyl branched derivatives. The primary function of CHCs is waterproofing; however, some specific CHCs serve ecological, behavioral, and biochemical functions. CHCs serve as sex pheromones in flies, precursors of contact sex pheromone in cockroaches, and nest-mate recognition signals in social insects. Diverse CHCs have also been used for taxonomic purposes in mosquitoes, termites and other insect species (Howard and Blomquist, 2005).

Considerable work has been done towards a sound understanding of the biosynthetic pathway of HCs in the fruit fly, *Drosophila melanogaster*. Briefly, CHC biosynthesis involves long-chain fatty acid synthesis, an elongation step to form very-long chain fatty acids, their conversion to aldehydes, and a final

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oxidative decarbonylation step (Chung et al., 2014; Howard and Blomquist, 2005; Qiu et al., 2012). Many enzymatic genes have been found to be involved in the formation of HCs. An acetyl-CoA carboxylase (*ACC*) (Parvy et al., 2012; Wicker-Thomas et al., 2015); two fatty acid synthases (*mFAS*^{CG3524}, *FASN*^{CG3523}) (Chung et al., 2014; Garrido et al., 2015; Wicker-Thomas et al., 2015); an elongase (*EloF*) (Chertemps et al., 2007; Wicker-Thomas et al., 1997); three desaturases (*Desat1*, *Desat2* and *DesatF*) (Chertemps et al., 2006; Fang et al., 2009; Labeur et al., 2002; Marcillac et al., 2005; Wicker-Thomas et al., 2009); a NADPH-cytochrome P450 reductase gene *CPR* and an oxidative decarbonylase P450 gene *CYP4G1* (Qiu et al., 2012).

Hundreds of multifunctional genes of the P450 superfamily have been found in the genomes of insects (Feyereisen, 1999, 2011; Nelson, 2009). Further, there are about 20–50 CYP4 genes that vary widely in each insect species (Feyereisen, 2011, 2012), while in the subfamily of CYP4G there are only one or very few CYP4G genes in most insect species. Cytochromes P450 were originally recognized to be involved in HC formation by converting aldehyde to the sex pheromone of HC with release of CO_2 in *vitro* in the house fly, *Musca domestica* (Reed et al., 1994, 1995). To date, the *D. melanogaster CYP4G1* and its orthologs, the *M. domestica CYP4G2* are the first two members of the CYP4G clan to be identified and functionally elucidated, encoding an oxidative decarbonylase that catalyzes aldehyde to HC (Qiu et al., 2012). The NADPH-cytochrome P450 reductase (*CPR*), a redox partner of *CYP4G1* and *CYP4G2*, has also been found to participate in the HC biosynthesis in *D. melanogaster* (Qiu et al., 2012). The CYP4G genes are conserved ortholog members across the Insecta, and the functions of CYP4G in all insects may be highly conserved. After the publication of the pea aphid genome (International Aphid Genomics, 2010) we searched the database and found a CYP4G gene, *CYP4G51*, and we speculate may be involved in aphid HC biosynthesis.

Aphids are regarded as major pests of agricultural crops, and cause huge economic losses worldwide both through direct feeding on phloem sap and as powerful vectors of plant viral pathogens. The traits of diverse species, super fecundity, and strong adaptation to variable environments make aphids very difficult to manage (Jaubert-Possamai et al., 2007; Sabater-Muñoz et al., 2006). Large-scale application of insecticides is not environmentally friendly and is therefore becoming increasingly unacceptable for aphid control (Sabater-Muñoz et al., 2006). The accessibility of the pea aphid genome sequence (International Aphid Genomics, 2010) has provided an opportunity to develop new strategies with molecular tools for aphid control.

RNA interference (RNAi) is the post-transcriptional gene silencing with high specificity, which can be triggered by double-stranded RNA (dsRNA). Delivery of exogenous dsRNA leads to sequence-specific suppression of gene transcript in the target organisms (Scott et al., 2013; Zhu, 2013). DsRNA mediated RNAi has revolutionized the entomological research, especially in the field of functional genomic study, and has also revealed great potential in insect pest management (Kim et al., 2015; Zhu, 2013). The roles of diverse proteins involved in various physiological courses have been unraveled through RNAi knockdown of the target genes in many insect species including the pea aphid (Dillen et al., 2016; Franco et al., 2016; Jaubert-Possamai et al., 2007; Mutti et al., 2006; Sapountzis et al., 2014; Will and Vilcinskas, 2015). In this study we conducted a molecular characterization of *Acyrthosiphon pisum CYP4G51* and performed RNAi of this gene. We discovered that both ingestion and injection of *CYP4G51* dsRNA caused a significant reduction in transcriptional expression of the target gene, and resulted in a decreased amount of both cuticular and internal HCs. Our results support the notion that *CYP4G51* is involved in water retention by modulating HC biosynthesis in the pea aphid.

2. Materials and methods

2.1. Pea aphid populations

Two pea aphid experimental morphs with strikingly different body colors, a red morph originally collected from Gansu province, China and a green morph kindly provided by Prof. Angela Douglas (Cornell University, USA), were used in this study. The green color colony was mainly used for RNAi treatments. Both colonies derived from a single respective parthenogenetic female were maintained at low density (no emergence of alate aphids) on broad bean seedlings (*Vicia faba* L. var. Jin-nong) in a climate chamber at 18 °C, ~70% relative humidity (RH) and a 16: 8 h (light: dark) photoperiod. In order to synchronize development and to harvest the third instar (L3) nymphs and adults, newborn offspring (the first instar or L1) produced by parthenogenetic adult females during a 12-h period were collected and placed on new plants. L3 nymphs and adults were obtained 6 d and 10 d after collection of L1 nymphs, respectively. The precise instar of nymphs was verified by examining the exuviae throughout the experiment.

2.2. Bioassay of desiccation resistance

For analysis of *CYP4G51* mRNA level, both L3 nymphs and adults were subjected to desiccation stress as described by Gibbs et al. (1997). Briefly, 30–40 aphids were transferred to an empty centrifuge vial (50 mL) and restricted to the lower half of the vial with an absorbent cotton stopper wrapped in gauze. Approximately 10 g of fresh allochroic silica gel (2.0–5.6 mesh, Ubao, Qingdao, China) was added on top of the stopper and the vial was sealed with double-layer of stretched Parafilm (Bemis Flexible Packaging, Neenah, WI, USA). Monitoring with a HOBO Pro v2 Data Logger (Onset Computer Corporation, Bourne, MA, USA) indicated that the RH of the closure of the vial drops to 5% within 60 min. The duration of the stress for L3 nymphs and adults was 24 and 40 h respectively. In control experiments, aphids were placed in vials with no desiccant and Parafilm, and exposed to a ~70% RH. All aphids were then subjected to RNA isolation, cDNA synthesis and quantitative PCR as described in Section 2.3 below.

For desiccation tolerance bioassay on aphids injected with dsRNA, ~20 ds*Muslta*- or ds*CYP4G51*-injected aphids (the fifth day after the first dsRNA injection) were subjected to desiccation (<5% RH) at either 18 °C or 25 °C for 24 h, and mortality was recorded. The surviving aphids were then individually transferred to the normal broad bean rearing condition. Mortality and fecundity were recorded at 24 h intervals for 7 days. Death was indicated by failure to right themselves or to move forward.

2.3. Tissue dissection, RNA isolation, cDNA synthesis and cloning

Various tissues or body parts were obtained by dissecting the parthenogenetic adults (the green morph) in 4 °C cold 0.01 M phosphate buffer saline. The gut and embryos were dissected out from abdomen followed by two rinses of DEPC-treated water. The abdominal cuticle was the cuticle without any internal embryos and gut, but with partial fat body attached. The three intact body parts of head, thorax and abdomen were obtained by dissecting whole adult aphids. All dissected samples were kept in a −80 °C freezer before the subsequent total RNA extraction.

Total RNA was isolated from the aphids of various ages or tissues using RNAiso plus reagents (Takara, Dalian, China), following the supplier's instructions. An aliquot of 800 ng of total RNA was used to synthesize first strand cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China). Gene specific paired primers (10 μM each) were designed to amplify a