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The dual roles of *Armigeres subalbatus* prophenoloxidase V in parasite melanization and egg chorion melanization in the mosquito *Ar. subalbatus*

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ABSTRACT

Phenoloxidases (POs) play key roles in various physiological functions in insects, e.g., cuticular sclerotization, wound healing, egg tanning, cuticle formation and melanotic encapsulation of pathogens. Previously, we identified five POs, designated As-pro-PO I–V, from the mosquito *Armigeres subalbatus* and demonstrated that the functions of As-pro-PO I, II and III, were associated with filarial parasite melanization, blood feeding and cuticle formation, respectively. In the present study, we delineate the dual functions of As-pro-PO V. We found that the level of *As-pro-PO V* mRNA in mosquitoes was significantly increased after microfilaria challenge or blood feeding, and decreased to normal level after oviposition. Knockdown of As-pro-PO V by dsRNA resulted in significant decreases in the degree of microfilaria melanization, egg chronic melanization rates and egg hatching rates in *Ar. subalbatus*. Further transfection and electrophoretic mobility-shift assays verified the *As-pro-PO V* gene might regulated by both AP-1, a putative immune-related regulatory element and CdxA, a developmental regulatory element. The binding of AP-1 and CdxA motif with mosquito nuclear extracts was significantly enhanced after microfilaria challenge and blood-feeding in *Ar. subalbatus*, respectively. These results indicate that As-pro-PO V is a critical enzyme that is required for both an effective melanization immune response and egg chorion melanization in this mosquito.

1. Introduction

The biosynthesis of melanin and melanotic material constitutes an important component in various physiological functions in insects, including cuticle sclerotization, wound healing, egg tanning and melanotic encapsulation of parasites (Christensen et al., 2005; Tsao et al., 2010). In insects, the key enzyme in the biosynthesis of melanin is phenoloxidase (PO). PO is a multiple copper containing oxidase that catalyzes the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (dopa) and the oxidation of ortho-diphenolic substances to their respective quinones that then polymerize to form melanin (Ashida and Yamazaki, 1990). POs are present as an inactive form, prophenoloxidase (PPO).

Genome analyses revealed that mosquitoes have more PPO-cording sequences than other non-blood-feeding insects. For example, the genomes of *Drosophila melanogaster* and *Manduca sexta* contain three PPO genes, respectively (Asada et al., 2003; Kanost et al., 2004), whilst 10 and 9 PPO genes are present in the genomes of *Aedes aegypti* and *Anopheles gambiae*, respectively (Christophides et al., 2002; Waterhouse et al., 2007). Although the genome of the mosquito *Armigeres subalbatus* has not been sequenced, we previously identified five PPOs, designated As-pro-PO I to V, from *Ar. subalbatus* (Tsao et al., 2009). The importance of PO paralogs in mosquitoes is not fully understood. Results of several studies showed that a blood meal induced the expression of multiple POs in mosquitoes, i.e., *An. gambiae* prophenoloxidase (AgPPO) 1, 2, 3, 4 *and* 9 (Müller et al., 1999; Marinotti et al., 2005; Amenya et al., 2010) and *Ae. aegypti* prophenoloxidase (AePPO) *2*, *4*, *5*, *6*, *9* and *10* (Kim et al., 2005). Multiple POs are also induced in response to microbial infection. Zou et al. (2008) demonstrated that AePPO *1*, *3*, *5* and *8* transcription was induced after bacterial and fungal infections in female *Ae. aegypti*. These results indicate that multiple POs can be involved in a specific mosquito physiology. On the other hand, evidence provided by

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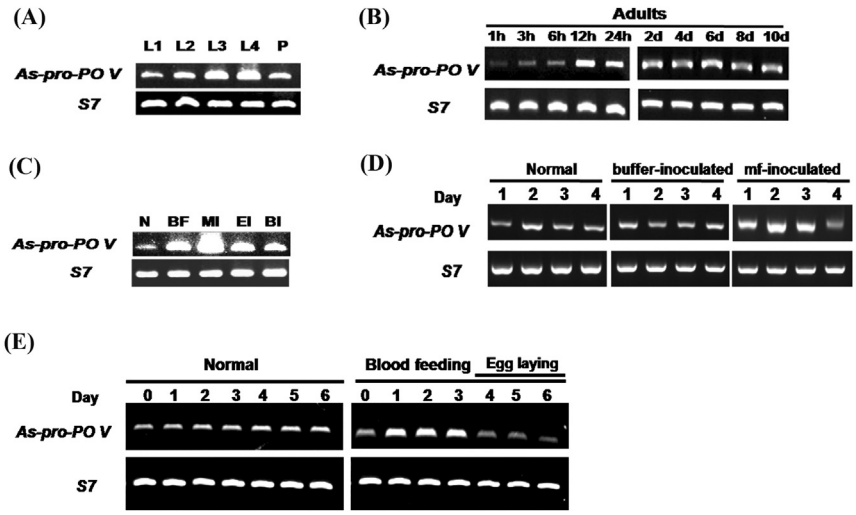

Fig. 1. RT-PCR analysis of the expression levels of *As-pro-PO V* in *Ar. subalbatus*. (A) *As-pro-PO V* transcription levels in larvae and pupae. L1: 1st stage larvae; L2: 2nd stage larvae; L3: 3rd stage larvae; L4: 4th stage larvae; P: pupae. (B) *As-pro-PO V* transcription levels in adults, 1–10 days after emergence. (C) *As-pro-PO V* transcription levels in mosquitoes at 48 h after blood feeding (BF), microfilariae inoculation (MI), *E. coli* inoculation (EI), buffer inoculation (BI) and N: naïve. (D) *As-pro-PO V* transcription levels in mosquitoes at various time intervals after buffer-inoculation and mf-inoculation. (E) *As-pro-PO V* transcription levels in mosquitoes at various time intervals after blood feeding. Mosquitoes were allowed to lay their eggs at the 4th day after blood feeding. S7 was used as a loading control.

gene knock-down experiments demonstrated that an individual PO can play a significant role in a specific physiological function. For example, Shiao et al. (2001) demonstrated that melanization of microfilariae (mf) in *Ar. subalbatus* is almost completely abolished by transducing with antisense RNA that is targeted to the copper–binding region of *As-pro-PO I*. They suggested that *As-pro-PO I* is an essential component for the melanization of parasites in mosquitoes. In addition, Tsao et al. (2010) demonstrated that knockdown of *As-pro-PO III* by double-stranded RNA in pupae resulted in the incomplete formation of nascent pupal endocuticle and pharate adult cuticle, i.e., significantly fewer cuticular lamellae were deposited and the helicoidal pattern of chitin microfibrils in lamellae was disorganized. They suggested that *As-pro-PO III* is required for cuticle formation during pupal morphogenesis in *Ar. subalbatus*.

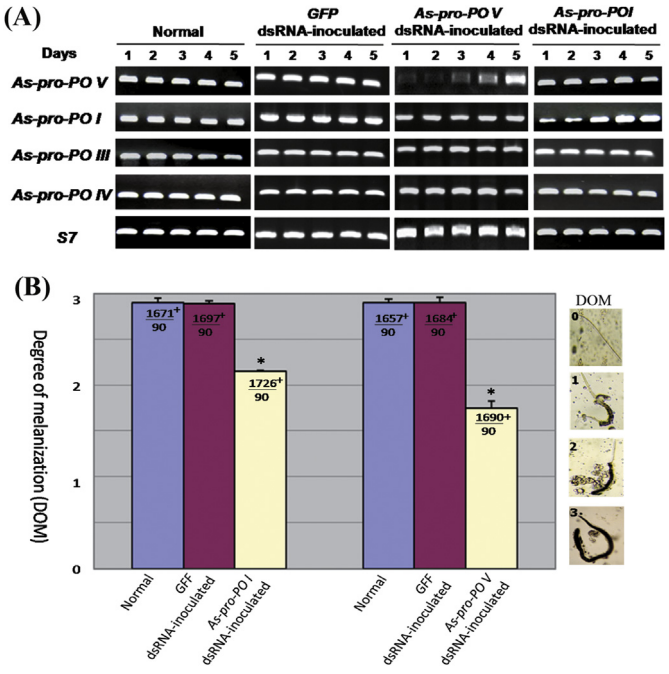

Fig. 2. Knockdown of *As-pro-PO V* transcription in *Ar. subalbatus* females reduces parasite melanization rates. (A) RT-PCR analysis of the transcription levels of *As-pro-POs* in naïve, *GFP* dsRNA-inoculated, *As-pro-PO V* dsRNA-inoculated, and *As-pro-PO I* dsRNA-inoculated *Ar. subalbatus*. (B) The degree of melanization of microfilariae (DOM) in *As-pro-PO I* dsRNA-inoculated and *As-pro-PO V* dsRNA-inoculated *Ar. subalbatus*. DOM (0–3): Two days after dsRNA inoculation, mosquitoes were challenged with microfilariae (mf). At three days post inoculation, mosquitoes were dissected to record the degree of melanization of mf in mosquitoes. This was based on a scale of 0 (unmelanized) to 3 (totally melanized) as described by Chen and Laurence (1987) (The right of the panels). +: The numbers indicate the total number of mf recovered and evaluated/the total number of mosquitoes dissected. All tests were repeated three times (*: $p < 0.05$).

2. Materials and methods

2.1. Biological materials

The source and maintenance of *Ar. subalbatus* was as previously described (Cho et al., 1998). *Dirofilaria immitis* microfilariae (mf)