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Wolbachia-induced paternal defect in *Drosophila* is likely by interaction with the juvenile hormone pathway

Chen Liu [a,1], Jia-Lin Wang [a,1], Ya Zheng [a], En-Juan Xiong [a], Jing-Jing Li [a], Lin-Ling Yuan [a], Xiao-Qiang Yu [a,b], Yu-Feng Wang [a,*]

[a] Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Science, Central China Normal University, Wuhan 430079, PR China
[b] School of Biological Sciences, University of Missouri-Kansas City, MO 64110, USA

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ABSTRACT

Wolbachia are endosymbionts that infect many insect species. They can manipulate the host's reproduction to increase their own maternal transmission. Cytoplasmic incompatibility (CI) is one such manipulation, which is expressed as embryonic lethality when *Wolbachia*-infected males mate with uninfected females. However, matings between males and females carrying the same *Wolbachia* strain result in viable progeny. The molecular mechanisms of CI are currently not clear. We have previously reported that the gene *Juvenile hormone-inducible protein 26* (*JhI-26*) exhibited the highest upregulation in the 3rd instar larval testes of *Drosophila melanogaster* when infected by *Wolbachia*. This is reminiscent of an interaction between *Wolbachia* and juvenile hormone (JH) pathway in flies. Considering that *Jhamt* gene encodes JH acid methyltransferase, a key regulatory enzyme of JH biosynthesis, and that methoprene-tolerant (Met) has been regarded as the best JH receptor candidate, we first compared the expression of *Jhamt* and *Met* between *Wolbachia*-infected and uninfected fly testes to investigate whether *Wolbachia* infection influence the JH signaling pathway. We found that the expressions of *Jhamt* and *Met* were significantly increased in the presence of *Wolbachia*, suggesting an interaction of *Wolbachia* with the JH signaling pathway. Then, we found that overexpression of *JhI-26* in *Wolbachia*-free transgenic male flies caused paternal-effect lethality that mimics the defects associated with CI. *JhI-26* overexpressing males resulted in significantly decrease in hatch rate. Surprisingly, *Wolbachia*-infected females could rescue the egg hatch. In addition, we showed that overexpression of *JhI-26* caused upregulation of the male accessory gland protein (Acp) gene *CG10433*, but not *vice versa*. This result suggests that *JhI-26* may function at the upstream of *CG10433*. Likewise, overexpression of *CG10433* also resulted in paternal-effect lethality. Both *JhI-26* and *CG10433* overexpressing males resulted in nuclear division defects in the early embryos. Finally, we found that *Wolbachia*-infected males decreased the propensity of the mated females to remating, a phenotype also caused by both *JhI-26* and *CG10433* overexpressing males. Taken together, our results provide a working hypothesis whereby *Wolbachia* induce paternal defects in *Drosophila* probably by interaction with the JH pathway *via* JH response genes *JhI-26* and *CG10433*.

1. Introduction

Wolbachia are intracellular microbes found frequently in wide variety of insects. It was estimated that up to 66% of all insect species harbor *Wolbachia* (Hilgenboecker et al., 2008). The successful spread of *Wolbachia* is thought to be attributed to efficient maternal transmission through the germline and manipulating the host reproduction that favors infected females.

Cytoplasmic incompatibility (CI) is the common reproductive modification induced by *Wolbachia* and results in lethality of embryos derived from crosses between *Wolbachia*-infected males and uninfected females or females carrying a different *Wolbachia* strain. However, females infected with the same *Wolbachia* strain can rescue the embryonic lethality associated with CI (Serbus et al., 2008; Werren et al., 2008). *Wolbachia*-mediated reproductive phenotype, especially CI, may be used as a unique tool for manipulating insect pest and vector populations, with potential

* Corresponding author. Tel.: +86 27 6786 7021; fax: +86 27 6786 1147.
E-mail address: yfengw@mail.ccnu.edu.cn (Y.-F. Wang).
[1] These authors contributed equally to this work.

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applications such as life shortening (McMeniman et al., 2009) and reduction of vector competence (Bian et al., 2010). Despite the biological and medical importance, the molecular mechanisms of CI are currently not clear, yet several evidences suggest that the modification of sperm by *Wolbachia* during spermatogenesis could be the main cause, since CI-induced defects are limited to paternal chromosomes (Serbus et al., 2008; Callaini et al., 1997; Landmann et al., 2009). Overexpression of either nonmuscle myosin II gene *zipper* or its downstream *l(2)gl* in *Wolbachia* free transgenic *Drosophila melanogaster* males results in paternal-effect lethality similar to the defects related to CI with regard to egg-hatch rates, though the presence of *Wolbachia* in the egg can not rescue the CI-like phenotype (Clark et al., 2006). This result favors a working hypothesis whereby *Wolbachia* induces paternal effects in sperm by manipulating the expression of key regulators of cytoskeletal activity during spermatogenesis. Landmann et al. have demonstrated that *Wolbachia*-infection delays the deposition of the replication-independent histone H3.3/H4 complex to the male pronucleus, which could explain the chromosome condensation and segregation defects observed in incompatible crosses (Landmann et al., 2009). Recently, Beckmann et al. detected a few *Wolbachia* proteins in the infected mosquito gonads and spermathecae, implying their roles as candidate CI effectors (Beckmann and Fallon, 2013; Beckmann et al., 2013). Our previous study shows that *Hira*, encoding one of the chaperones of H3.3, is significantly decreased in transcripts in *Wolbachia*-infected male flies that induce strong CI when compared to *Wolbachia*-uninfected males (Zheng et al., 2011a). Additionally, *Hira* mutated male flies may also result in significantly decreased hatch rates, and *Wolbachia*-infected females can rescue the reduced hatch rates. These strongly mimic the defects associated with CI, suggesting that HIRA could be correlated to the abnormal deposition of H3.3 to the paternal chromosome in CI embryos. To understand the molecular mechanisms of *Wolbachia*-induced CI, we have compared the transcriptional profiles by microarray and identified 167 upregulated genes ($\geq$1.5 fold changes, q-value $<$5%) in the 3rd instar larval testes when infected by *Wolbachia*, of which *CG3767-RA* (*Juvenile hormone-inducible protein 26*, *JhI-26*) shows the highest upregulation (about 10 fold) relative to uninfected ones (Zheng et al., 2011b). This result indicates that *Wolbachia* may interact with the juvenile hormone (JH) signaling pathway and *JhI-26* might play an important role in male reproduction.

JH is important for regulating both metamorphosis and reproduction in insects (Flatt et al., 2005; Jindra et al., 2013). The effects of JH on female reproduction are well documented. For example, JH regulates oocyte maturation and reproductive behaviour (Riddiford, 2012). However, in male insects, JH effects on reproduction are poorly understood. No role for JH during *D. melanogaster* spermatogenesis has been reported. But it has indeed been shown to function in accelerating the maturation of male accessory gland (MAG) and stimulating the secretion of male accessory gland proteins (Acps) (Gold and Davey, 1989; Wilson et al., 2003; Parthasarathy et al., 2009). Acps are secreted from MAG and are transferred along with sperm into the female reproductive tract during mating. These proteins initiate a series of behavioral and morphological changes to promote the reproductive success of the inseminated females (Avila et al., 2011). Experiments in *D. melanogaster* indicate that Acps increase egg laying (Heifetz et al., 2005), food uptake (Carvalho et al., 2006) and decrease propensity to remating (Yapici et al., 2008; Yang et al., 2009). However, it is not clear how JH regulates the Acps function in insects.

JhI-26 is a JH-inducible gene and its expression can be triggered rapidly and specifically by JHIII or its agonist methoprene (Dubrovsky et al., 2000). Its biological function has not been reported. Recently, *JhI-26* has been identified as a sperm protein in *D. melanogaster* (Wasbrough et al., 2010), which supports our hypothesis that *JhI-26* may function in male fertility.

In order to investigate whether *Wolbachia* are involved in the JH signaling, we first compared the expression levels of JH acid methyltransferase gene *Jhamt* and methoprene-tolerant gene *Met* between *Wolbachia*-infected and uninfected *D. melanogaster*. We found that the expression levels of both *Jhamt* and *Met* were significantly increased in *Drosophila* testes when infected by *Wolbachia*, indicating that *Wolbachia* affected JH synthetic process and signaling pathway. Overexpression of *JhI-26* in males led to significantly higher embryonic lethality, and *Wolbachia*-infected females could rescue this defect. Further, *JhI-26* overexpression upregulated the expression of one Acp gene *CG10433*, which also showed upregulation in our previous microarray analysis (Zheng et al., 2011b). Increasing *CG10433* levels in males could also lead to higher embryonic lethality. Finally, we found that both *JhI-26* and *CG10433* overexpressing males may notably reduce the receptivity of mated females to remating, similar to *Wolbachia*-infected males. These results suggest that *Wolbachia* might induce paternal defects by interaction with the JH signaling pathway with consequences on *JhI-26* and *CG10433* expression.

2. Materials and methods

2.1. Drosophila and Wolbachia strains

Fruit flies were reared in a standard cornmeal/yeast diet at 25 °C and under non-crowded conditions (around 200 eggs per 50 ml vial of media in 150 ml conical flask) (Yamada et al., 2007). The *w*Mel *Wolbachia* infected *D. melanogaster*, designated as Dmel *w*Mel, was kindly provided by Professor Scott O'Neill (Monash University, Australia). Cured Dmel *w*Mel was designated as Dmel T, which was generated by tetracycline treatment as described previously (Hoffmann et al., 1986) and confirmed to be *Wolbachia*-free by PCR with primers for *Wolbachia* surface protein gene (*wsp*) (Table 1). The cured flies were maintained for over 5 generations in normal tetracycline-free medium to eliminate any effects of residual tetracycline. ActGal4 flies were from Dr. Nathalie Dostatni at Curie Institute, Paris, France. NosGal4 flies were kindly provided by Professor Zhaohui Wang at Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

2.2. Gene expression assay

Quantitative RT-PCR (qRT-PCR) was performed to investigate the relative gene expression level. Total RNA was extracted using Trizol (Invitrogen). DNA contamination was removed with RQ1 DNase (Promega). The first-strand cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Invitrogen) and oligo dT15 primer (Takara) at 42 °C for 1.5 h. Specific primers for tested genes were designed based on sequences from flybase database (Table 1). QPCR was performed using a Miniopticon system (BioRad) with a Platinum SYBR Green qPCR SuperMix (Takara) as described previously (Zheng et al., 2011b). The qPCR cycling program was 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 52–61 °C (based on various primers) for 20 s and 72 °C for 20 s, and then a melting curve was constructed from 55 °C to 98 °C. The relative expression of each gene was calibrated against the reference gene (*rp49*) using $2^{-\Delta CT}$ ($\Delta C_T = C_{T,\ \text{target gene}} - C_{T,\ rp49}$).

2.3. Generation of transgenic fly strains and genetics

RNA extracted from Dmel T was reverse transcribed into cDNA. The specific primers (Table 1) were used to amplify the coding sequences of *JhI-26-RA* and *CG10433-RA* by PCR. After digestion