FKBP46 plays a role in promoting the egg diapause of the migratory locust, *Locusta migratoria*

Zhang Daogang Chen Jun Cui Dongnan Li Shuang Pan Fan Zhu Kaihui
Tu Xiongbing* Zhang Zehua*

(State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China)

Abstract: The aim of the present study was to verify the function of FK506 binding protein 46 (FKBP46) in regulating the diapause of the migratory locust, *Locusta migratoria*. *FKBP46* was cloned from *L. migratoria* and the double-stranded RNA of *FKBP46* was synthesized, which was then injected into *L. migratoria* female adults to observe the changes in calcineurin (CaN) activity and the rate of egg diapause. The results showed that RNA interference of *FKBP46* significantly decreased egg diapause rates from 97.44% to 78.59% under short photoperiods. Under long photoperiod, all *L. migratoria* eggs hatched after *FKBP46* RNA interference, with no difference from the control group. Meanwhile, CaN activity was up-regulated from 0.24 U/g to 0.29 U/g in the hind leg and from 0.21 U/g to 0.30 U/g in the fat body, respectively, after *FKBP46* gene silencing. These results suggested that *FKBP46* negatively regulated CaN to affect *L. migratoria* diapause under short photoperiod. This regulatory gene is probably an ideal target for RNAi-based diapause control of the migratory locust.

Key words: *Locusta migratoria*; diapause; *FKBP*; calcineurin activity; RNA interference

FKBP46对飞蝗卵滞育具有促进作用

张道刚 陈 俊 崔栋楠 李 霜 潘 凡 朱凯辉 涂雄兵* 张泽华*

(中国农业科学院植物保护研究所，植物病虫害生物学国家重点实验室，北京 100193)

摘要：为验证FK506结合蛋白46（FK506 binding protein 46，FKBP46）在调节飞蝗*Locusta migratoria*滞育中的作用，首先从飞蝗中克隆*FKBP46*，然后合成双链RNA，将其注入到飞蝗雌成虫体内进行干扰，观察干扰后钙调磷酸酶（calcineurin，CaN）的活性和卵滞育率的变化。结果显示，短日照条件下，RNA干扰*FKBP46*后飞蝗卵滞育率显著降低，由97.44%降至78.59%；在长日照条件下，RNA干扰*FKBP46*后，所有飞蝗卵均成功孵化，与对照组无显著差异；*FKBP46*基因沉默后，飞蝗后足中CaN活性分别从0.24 U/g上调至0.29 U/g，脂肪体中CaN活性从0.21 U/g上调至0.30 U/g。表明在短日照条件下*FKBP46*负调控CaN影响飞蝗滞育，*FKBP46*的沉默效应表明该调控基因可能是基于RNA干扰的飞蝗滞育控制的理想靶点。

关键词：飞蝗；滞育；*FKBP*；钙调磷酸酶活性；RNA干扰

Immunophilin is a type of cell receptor protein that binds to the immunosuppressant cyclosporine A (CsA), FK506 and rapamycin. According to the immunosuppressor, the immunophilin family is divided into

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* 通信作者（Authors for correspondence），E-mail：xbtu@ippcaas.cn，zhangzehua@caas.cn

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two categories: one class called cyclophilin (CyP) that binds to cyclosporin A; another class called FK506 binding protein (FKBP) which specifically binds to FK506 and rapamycin (Schreiber, 1991; Fruman et al., 1994). FKBP forms a FK506-FKBP complex with FK506 and inhibits calcineurin (CaN) activity (Sigal & Dumont, 1992). CaN, a conserved protein phosphatase 2B (PP2B), is a calmodulin-dependent serine/threonine protein phosphatase (Lee et al., 2013). CaN is involved in a variety of developmental and cellular processes in different organisms and in different type cells, such as T cell activation, growth and differentiation of skeletal muscle and heart muscle (Kingsbury & Cunningham, 2000). The activity of CaN in fertilized eggs is critical for the onset of vertebrate embryo development (Nishiyama et al., 2007). In addition, CaN is also involved in the diapause process of silkworm embryos (Hsieh & Gu, 2019). The relationships between FKBP and CaN in development and diapause process of silkworm embryos prompted us to study the effects of FKBP on diapause.

A common feature of FKBPs is the peptidyl-prolyl cis-trans isomerase (PPIase) activity, which catalyzes the conformation of the N-terminal peptide bond of a proline residue in a polypeptide or protein substrate convert to trans from cis (Fischer et al., 1984; Harding et al., 1989; Siekierka et al., 1989). In addition to PPIase enzymatic activity, FKBPs also act as molecular chaperones, providing binding sites for interactions between proteins (van Duyne et al., 1991). In addition, FKBPs are involved in many life processes such as apoptosis, cell cycle progression, calcium ion release, nucleic acid binding, and transport of macromolecular receptor complexes (Somarelli & Herrera, 2007). For example, in early pregnancy, the FKBP52-peroxiredoxin-6 (FKBP52-PRDX6) signal in the uterus is able to counteract adverse reactions due to oxidative stress (Hirota et al., 2010). FKBP51 acts as a synergistic chaperone of heat shock protein 90 (HSP90), ensuring proper protein folding under protein toxicity (Rein, 2016). A weak interaction between HSP47 and FKBP65 confers mutual molecular stability and also allows for a synergistic effect during collagen folding (Ishikawa et al., 2017). FKBPs have been shown to interact with numerous receptor signaling pathways in different organisms, the CaN signaling pathway is one of them. The CaN signaling pathway is composed of calcium channels (Cch1), transporters (Mid1), a calcium sensory protein (calmodulin, CaM), a calcium-calmodulin dependent phosphatase (calcineurin), and calcineurin interacting proteins (*i. e.* cyclophilins, FKBP, CyP) (Park et al., 2019). CaN is a heterodimeric complex consisting of a catalytic A (CnA) subunit and a regulatory B subunit (CnB) (Steinbach et al., 2007). CaM can detect changes in the cytoplasmic Ca^{2+} concentration. When the Ca^{2+} concentration is low, the CnB subunit interacts with CaM binding domain, while the autoinhibitory domain blocks the catalytic site of the CnA subunit; when the Ca^{2+} concentration is high, Ca^{2+} bind to CaM and CnB subunit, leading to conformational changes of CnB subunit. CaM binds to CnA subunits and releases from the autoinhibitory domain from the substrate binding site, thereby activating CaN dephosphorylation target (Rusnak & Mertz, 2000). FKBP inhibits the CnA and CnB subunits by binding to FK506, thereby inhibiting the calcium signal cascade. Most of the previous research on FKBP focused on the field of immunity, its relationship with insect development and diapause is unclear.

The migratory locust, *Locusta migratoria*, is an insect exhibiting facultative diapause. Diapause is of great significance to the continuation of insect species. It can result in a delay of parturition to ensure optimal nutritional and seasonal environments for survival (Renfree & Shaw, 2000). The physiological and biochemical indicators in insects will change with the environmental factors. Molecular mechanisms related to insect diapause have been widely studied, such as the hormone (Denlinger et al., 2012), circadian clock (Koštál, 2011; Saunders, 2020) and energy control (Hahn & Denlinger, 2007; 2011). However, the role of FKBPs in diapause regulation is rarely reported. In the early stage, we performed proteomic sequencing and analysis on diapause and non-diapause eggs of migratory locusts, and found that the protein expression level of FK506 binding protein 46 (FKBP46) in diapause eggs and non-diapause eggs was significantly

different. To test the function of FKBP46 on diapause, *FKBP46* dsRNA was injected into the migratory locust to study the effect of *FKBP46* gene on CaN activity and diapause rate of offspring. This study provides a new idea for the study of the diapause mechanism of locust by detecting the effects of FKBP46 on CaN activity and diapause rate of locust, so as to provide theoretical support for the outbreak of locust plague.

1 Materials and Methods

1.1 Materials

Insect materials: Eggs of *L. migratoria* were collected in Cangzhou City, Hebei Province, and were continuously raised for many generations in the Laboratory of Institute of Plant Protection, Chinese Academy of Agricultural Sciences. The eggs of *L. migratoria* hatched in an artificial climate box under the conditions of 30 ℃ and relative humidity of 60%. The adults within 48 h after eclosion were used for RNAi treatment. The wheat seedlings after germination for one week were used to feed *L. migratoria*, the wheat seeds called Jimai22 were bought online.

Medium: LB liquid medium is composed of tryptone 10 g, yeast extract 5 g, NaCl 10 g, set the volume to 1 L with deionized water; LB solid medium is composed of tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar powder 15 g, set the volume to 1 L with deionized water.

Reagents and instruments: TRIcom Reagent, Zymo Research, America; PrimeScript™ 1st strand cDNA Synthesis Kit, pMD19-T vector, TaKaRa, Japan; 1-5™ 2×High Fidelity Master Mix, MCLAB, America; TIANgel Midi Purification Kit, TIANGEN, China; Biomiga EZgene™ Plasmid Miniprep Mit, BIOMIGA, America; T7 RiboMAX™ Express RNAi System Kit, Promega, America; Insect CaN ELISA Kit, Quanzhou Kenuodi Biotechnology Co., Ltd., Quanzhou, China; all other reagents were domestic analytical pure. ABI 7500 real-time PCR system, ABI, America; Trans1-T1 strain of *Escherichia coli*, TransGen Biotech, China; Bester® SybrGreen qPCR Mastermix, DBI® Bioscience, Germany; HPX-9052 MBE digital display electric heating incubator, Shanghai Boxun Industrial Co., Ltd., China; DYCP-32B agarose horizontal electrophoresis system, Beijing Liuyi Instrument Factory, China; 2X-2020D Gel imaging analysis system, Shanghai Zhixin Instrument Technology Co., Ltd., China; ETC-811 PCR instrument, Beijing Dongsheng Innovation Biotechnology Co., Ltd., China; 10 μL micro syringe, BURKARD SCIENTIFIC Technology Company, Britain; PRX-250B-30 artificial climate box, Haishu Saifu Experimental Instrument Factory, Ningbo, China; Nano-300 photometer, Hangzhou ALLSHENG Instrument Co., Ltd., China.

1.2 Methods

1.2.1 Cloning of *FKBP46* gene from *L. migratoria*

A third instar nymph of *L. migratoria* was dissected and the internal organs were remove. The remaining tissue was used for the extraction of total RNA. TRIcom Reagent was used to extract total RNA. Total RNA was isolated according to the manufacturer's protocol. The quality was checked on a Nano-300 photometer with $A_{260\ nm}/A_{280\ nm}$ between 1.9 and 2.0, whereas the reliability of RNA was confirmed on 1% agarose gel, which gave three clear bands. cDNA was synthesized according to the PrimeScript™ 1st strand cDNA Synthesis Kit. By analyzing transcriptome of *L. migratoria*, the sequence of FKBP46 gene was obtained and primers were designed by DNAMAN 6.0 software. Using cDNA of *L. migratoria* as template, the *FKBP46* gene was amplified by primers *FKBP46*-1F(5′-GCTGTTGCGTTGGTTACATC-3′)/*FKBP46*-1R(5′-CCAGAACTCTCATTCAGTCACA-3′). 50 μL PCR reaction system: cDNA 2 μL, 2×High Fidelity Master Mix 25 μL, forward and reverse primers 2 μL each, ddH_2O 19 μL. The amplification procedure is as follows: 95 ℃ predenaturation 5 min, 95 ℃ denaturation 15 s, 57 ℃ annealing 10 s, 72 ℃ extension 30 s, 72 ℃ final extension 8 min. The obtained PCR product was purified by TIANgel Midi Purification Kit and was connected to the 1 μL pMD19-T vector, 6 μL solution and 3 μL DNA to incubate at room temperate for 6 h. Then the recombinant was transformed into the Trans1-T1 strain of *E. coli*. Add 500 μL LB liquid medium. Then shake at 200 r/min and 37 ℃ for 2 h. 100 μL of the above bacterial solution was applied to LB solid medium which include 1‰ ampicillin. The medium was

incubated at 37 ℃ for 12 h. The recombinant colonies were transferred into LB liquid medium with 1‰ ampicillin, and shaken for 3–6 h at 37 ℃, then took the medium for PCR templet to verify the product size as expected. All primers used in this experiment were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

1.2.2 dsRNA synthesis of *FKBP46* gene

Recombinant plasmid including *FKBP46* gene fragment cloned in 1.2.1 was extracted by using Biomiga EZgene™ Plasmid Miniprep Mit. Using the recombinant plasmid as template, the *FKBP46* gene of *L. migratoria* was amplified by primers *FKBP46*-2F(5′-TAATACGACTCACTATAGGTAGACGACG-ACGATGTTGA-3′)/*FKBP46*-2R(5′-TAATACGACT-CACTATAGGTGGAGTATTGGCACCTTTGT-3′).

50 μL PCR reaction system: cDNA 2 μL, 2×High Fidelity Master Mix 25 μL, forward and reverse primers 2 μL each, ddH_2O 19 μL. The amplification procedure is as follows: 95 ℃ pre-denaturation 5 min, 95 ℃ denaturation 15 s, 57 ℃ annealing 10 s, 72 ℃ extension 10 s, 72 ℃ final extension 8 min. The PCR amplified products were purified with TIANgel Midi Purification Kit, and the quantification of purified products was performed by a Nano-300 photometer. *FKBP46* dsRNA was synthesized using the T7 RiboMAX™ Express RNAi System Kit. *FKBP46* dsRNA concentration was detected by a Nano-300 photomter and the concentration was adjusted to 1 μg/μL.

1.2.3 RNAi efficiency

Female adults within 48 h after eclosion were injected with 10 μL *FKBP46* dsRNA (1 μg/μL) between the third and fourth abdominal segments of *L. migratoria*. Double-distilled water (ddH_2O) as control was injected into similarly prepared individuals. There were 25 female adults per repetition with a total of 75 individuals for either photoperiod treatment. For non-diapause *L. migratoria* in the experiment, the photoperiod was 16 L∶8 D, the temperature was 27 ℃ and the relative humidity was 60%. To induce diapause of *L. migratoria*, the photoperiod was 10 L∶14 D, the temperature was 27 ℃ and the relative humidity was 60%. Hind legs and fat bodies were acquired by dissecting the whole bodies of dsRNA-injected and control group's adults after 36 h. The efficiency of RNAi-mediated knockdown at different tissues of *L. migratoria* was determined with quantitative real-time PCR (qRT-PCR).

Total RNA was extracted from hind leg and fat body samples using TRIcom Reagent and the total RNA concentration was estimated by a Nano-300 photometer. 5 μL of total RNA was reverse-transcribed with PrimeScript™ 1st strand cDNA Synthesis Kit. To evaluate RNAi efficiency, primers *FKBP46*-3F (5′-GACAGTGATGAGGAAATGGAAG-3′)/*FKBP46*-3R (5′-GTGGGAGTGTTTGGAGTTGC-3′) were used to amplify endogenous *FKBP46* gene on the ABI 7500 real-time PCR system. qRT-PCR was performed with the Bester® SybrGreen qPCR Mastermix. 20 μL PCR reaction system: cDNA 1 μL, qPCR Mastermix 10 μL, forward and reverse primers 1 μL each, ddH_2O 7 μL. The amplification procedure is as follows: 95 ℃ denaturation 15 s, 60 ℃ annealing and extension 1 min. *Actin* gene was used as reference gene, the reference primers were *actin*-F(5′-GTTACAAACTGGGACGA-CAT-3′)/*actin*-R(5′-AGAAAGCACAGCCTGAATA-G-3′). A total of three technical replicates were set. The relative mRNA level of *FKBP46* was calculated by $2^{-\Delta\Delta Ct}$ method. $\Delta\Delta Ct = (Ct_{\text{FKBP46}}$ for treatment$-Ct_{\text{actin}}$ for treatment$)-(Ct_{\text{FKBP46}}$ for control$-Ct_{\text{actin}}$ for control).

1.2.4 Detection of CaN activity

Previous study showed that FK506 is a natural product that inhibits calcineurin signaling (High, 1994). The detection and measurement of CaN activities in tissues of ds*FKBP46*-treated and control locusts under long and short photoperiod were verified. Hind legs and fat bodies of three whole bodies randomly selected from dsRNA-injected and control group's adults after 36 h were used. CaN activity was detected using Insect CaN ELISA Kit according to the manufacturer's protocol.

1.2.5 Diapause rate

Previous study showed that *FKBP46* is a differentially gene between diapause eggs and non-diapause eggs, so diapause rate was detected to verify the function of FKBP46. Except for RNAi treatment and the detection of CaN activity, the remaining locusts were reared at 28 ℃, until laying eggs. The number of hatched nymph of locusts D_1 and the unhatched

eggs D_2 were counted, namely diapause rate= $D_1/(D_1+D_2)\times100\%$.

1.3 Statistical analysis

Statistical analyses were performed using SPSS 16.0 software. Independent-sample *t* test was used for mRNA levels, enzyme activities and diapause rate.

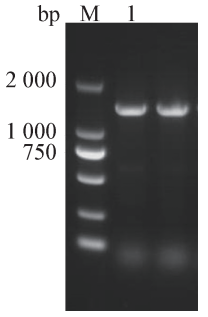
2 Results

2.1 dsRNA synthesis of *FKBP46* gene

The *FKBP46* gene was amplified on the basis of the sequence of *L. migratoria* transcriptome. The PCR amplified fragment was 1 500 bp (Fig. 1). The dsRNA of *FKBP46* gene was synthesized according to the procedure of the T7 RiboMAX™ Express RNAi System Kit and the expected size of *FKBP46*'s dsRNA is 595 bp. The size of the dsRNA was checked by 1% agarose gel electrophoresis (Fig. 2). The concentration of dsRNA was checked by Nano-300 photometer and adjusted to 1 000 ng/μL.

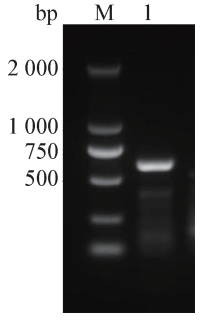
2.2 RNAi efficiency

To verify the function of FKBP46 in regulating *L. migratoria* diapause, dsRNA of *FKBP46* was synthesized and subsequently injected into female adults of *L. migratoria* for RNAi-mediated knockdown of *FKBP46* under long and short photoperiods, followed by confirming RNAi efficiency via qRT-PCR. The results showed that the mRNA level of *FKBP46* gene in hind leg and fat body was significantly lower in treatment than that in the control under long and short photoperiods ($P<0.05$, Fig. 3).



M: Marker; 1: PCR fragment of *FKBP46* gene.

Fig. 1 Electrophoresis pattern of *FKBP46* by PCR



M: Marker; 1: dsRNA of *FKBP46*.

Fig. 2 Electrophoresis of synthesized dsRNA

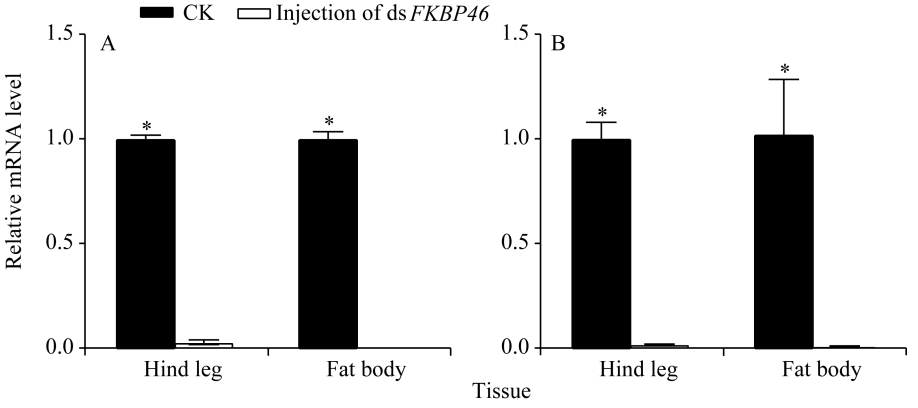


Fig. 3 Relative mRNA levels of *FKBP46* in *Locusta migratoria* under long (A) **and short photoperiod** (B) **after *FKBP46* RNAi**

Data are mean±SD. * indicates significant difference between CK and treatment used by *t* test ($P<0.05$).

2.3 CaN activity changes after *FKBP46* knockdown

CaN activity in hind leg and fat body of *FKBP46*-knockdown group was significantly higher than that of the control under long photoperiod ($P<0.05$), the activity was respectively up-regulated from 0.18 U/g to 0.32 U/g in hind leg, 0.15 U/g to 0.32 U/g in fat body (Fig. 4-A). Similarly, CaN activity in *FKBP46*-knockdown treatment was also significantly higher than that of the control under short photoperiod (Fig. 4-B), and the activity was respectively up-regulated from 0.24 U/g to 0.29 U/g in hind leg and 0.21 U/g to 0.30 U/g in fat body after *FKBP46* gene

silencing. The results indicated that FKBP46 negatively regulated CaN.

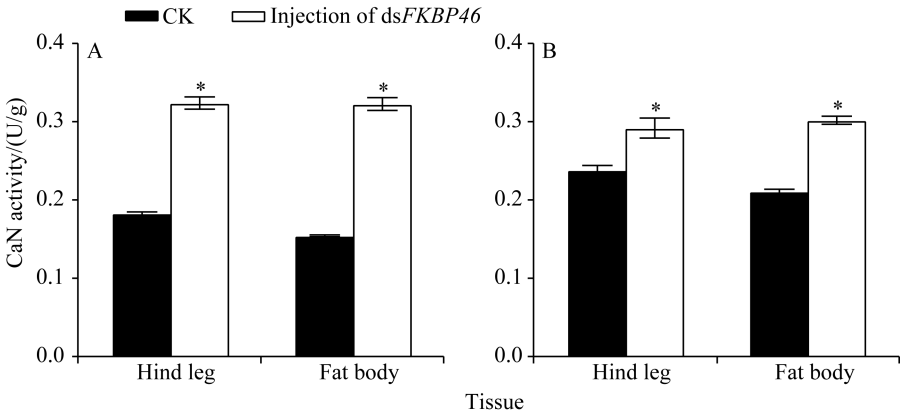


Fig. 4 Calcineurin activities in *Locusta migratoria* under long (A) **and short photoperiod** (B) **after *FKBP46* RNAi**

Data are mean±SD. * indicates significant difference between CK and treatment used by *t* test (P<0.05).

2.4 Diapause rate

Under long photoperiod, injecting with ds*FKBP46* had no effect on diapause rate of locust eggs. However, under short photoperiod, diapause rate of the individuals injected with ds*FKBP46* was 78.59%, which was significantly lower than control 97.44% (P< 0.05). It suggested that maternal FKBP46 could promote the egg diapause under short photoperiod conditions (Fig. 5).

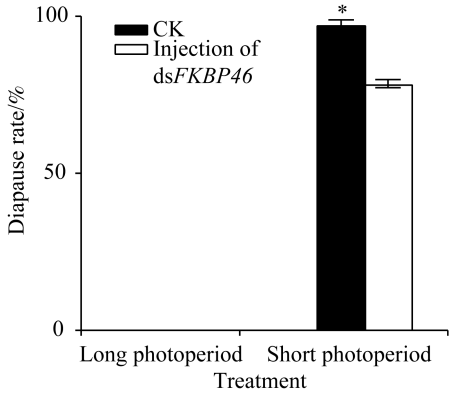


Fig. 5 Diapause rate of *Locusta migratoria* under long and short photoperiod after *FKBP46* RNAi

Data are mean±SD. * indicates significant difference between CK and treatment by *t* test (P<0.05).

3 Discussion

A proteomic study previously revealed the differential expression of the *FKBP46* gene in diapause *vs.* non-diapause eggs. It is supposed that FKBP46 may function in locust diapause. RNAi is a mature technology in *L. migratoria* (Guo et al., 2011; Ma et al., 2011; Wu et al., 2012). In this paper, RNAi-mediated knockdown of *FKBP46* was performed to investigate the effect of FKBP46 on *L. migratoria* diapause. It is found that RNAi-mediated knockdown of *FKBP46* in mature females ultimately decreased the extent of diapause in offspring eggs. The result showed that FKBP46 can induce diapause in locust. Because of the special relationship between FKBP and CaN, FKBP46 was likely to affect locust diapause through CaN under short photoperiod.

FK506 acts with FKBP to inhibit CaN activity (Liu et al., 1991). In immune cells, the immunophilin-immunosuppressive complex interacts with CaN to repress the phosphorylation signaling pathway during the expression of immune-related genes, producing immunosuppressive effects (Sigal & Dumont, 1992). There is a trade-off between immune defense and reproduction in insects (Schwenke et al., 2016). In *Drosophila melanoglia*, CaN appears to promote induction of innate immune responses and plays a key role in controlling systemic energy and body weight homeostasis (Dijkers & O'Farrell, 2007; Pfluger et al., 2015). The high expression pattern of CaN in the early embryo of *D. melanogaster* suggests that CaN plays an important role in embryonic development (Brown et al., 1994). In *Caenorhabditis elegans*, CaN signal can regulate lipid decomposition, development, behavior, aging and other life processes (Lee et al., 2013; Wang et al., 2017). In addition, CaN is differentially expressed between diapause eggs and develop-

mental eggs of *Bombyx mori*, and these changes may be related to the diapause process of silkworm embryos (Hsieh & Gu, 2019). The CaN activity after *FKBP46* RNAi is all up-regulated in different tissues, which is consistent with previous researches.

FKBPs play a significant role in development of mammals and plants (Breiman & Camus, 2002). Previous study showed that FKBP12 is important for expression of the cardiomyocyte phenotype and for regulation of contractile function during chick embryonic development (Obata et al., 2001). The expression pattern of the *FKBP12* gene during chick embryonic development suggests that FKBP12 may play a unique role in cardiogenesis (Yazawa et al., 2003). The results showed that diapause rate was lower in *FKBP46* RNAi treatment than that of control under short photoperiod, with 100% hatching rate under the long photoperiod for both treatment and control groups. The results suggested that FKBP46 may be involved in the induction process of diapause under the short photoperiod rather than under the long photoperiod. Future researches may explore other factors and genes that affect diapause in migratory locusts and focus on the specific mechanism of FKBP regulating diapause through the CaN signaling pathway.

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