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Molecular Characterization and Expression Profiles of *Cryptochrome* Genes in a Long-Distance Migrant, *Agrotis segetum* (Lepidoptera: Noctuidae)

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Abstract

Cryptochromes act as photoreceptors or integral components of the circadian clock that involved in the regulation of circadian clock and regulation of migratory activity in many animals, and they may also act as magnetoreceptors that sensed the direction of the Earth's magnetic field for the purpose of navigation during animals' migration. Light is a major environmental signal for insect circadian rhythms, and it is also necessary for magnetic orientation. We identified the full-length cDNA encoding As-CRY1 and As-CRY2 in *Agrotis segetum* Denis and Schiffermaller (turnip moth (Lepidoptera: Noctuidae)). The DNA photolyase domain and flavin adenine dinucleotide-binding domain were found in both *cry* genes, and multiple alignments showed that those domains that are important for the circadian clock and magnetosensing were highly conserved among different animals. Quantitative polymerase chain reaction showed that *cry* genes were expressed in all examined body parts, with higher expression in adults during the developmental stages of the moths. Under a 14:10 (L:D) h cycle, the expression of *cry* genes showed a daily biological rhythm, and light can affect the expression levels of *As-cry* genes. The expression levels of *cry* genes were higher in the migratory population than in the reared population and higher in the emigration population than in the immigration population. These findings suggest that the two *cryptochrome* genes characterized in the turnip moth might be associated with the circadian clock and magnetosensing. Their functions deserve further study, especially for potential control of the turnip moth.

Key words: *Agrotis segetum*, cryptochrome, magnetoreceptor, migration, photoreceptor

Cryptochromes (CRYs) are widespread in nature and have been found in many plants and animals. They are ultraviolet (UV)-A/blue light photoreceptors and belong to the photolyase/cryptochrome family (Todo et al. 1996, Cashmore et al. 1999, Sancar 2003, Müller and Carell 2009). They act as integral components of circadian clocks in animals (Miyamoto and Sancar 1998, Haque et al. 2002, Rubin et al. 2006, Tomioka and Matsumoto 2010). Based on the roles of CRYs in the regulation of circadian clocks in animals, CRYs have been classified into two categories: *Drosophila*-like type 1 Cry (CRY1-d) and mammal-like type 2 Cry (CRY2-m; Yuan et al. 2007, Zhu et al. 2008). CRY1 acts as a circadian photoreceptor involved in light-mediated entrainment of the circadian clock, whereas CRY2 acts in the negative feedback loop of the circadian oscillator by inhibiting CLOCK/BMAL1-driven transcription (Emery et al. 1998,

Krishnan et al. 2001, Gegear et al. 2008, Nießner et al. 2016). CRYs are known to play a crucial role in generating and regulating circadian rhythms in animals. Light is one of the major environment signals for synchronizing circadian rhythm to different environmental conditions of *Drosophila* (Diptera: Drosophilidae) and other insects (Dubruille and Emery 2008).

Another, CRYs were proposed as potential magnetoreceptors by Ritz et al. (2000) to explain the mechanism by which migratory birds are able to sense the direction of the Earth's magnetic field for the purpose of navigation during their migration. Recent reports show that CRYs are associated with the sensing of magnetic fields in several species. CRYs act as receptor molecules for directional information from the Earth's magnetic field during the migration of European robins and garden warblers (Heyers et al. 2007). Domestic

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chickens have the same type of magnetic compass mechanism as European robins (Nießner et al. 2011). CRYs are also essential for light-dependent sensing of magnetic fields by *Drosophila* (Gegear et al. 2008, Yoshii et al. 2012). So far, CRYs remain the best candidate for the radical-pair magnetoreceptors during animals' migration (Mouritsen et al. 2004, Phillips et al. 2010, Maeda et al. 2012, Yoshii et al. 2012, Dodson et al. 2013, Nohr et al. 2017).

Migration is a special type of animal movement and is an essential component of the life history and ecological niche of the organism. Migrating animals are found in all major branches of the animal kingdom, including invertebrate and vertebrate species (Dingle and Drake 2007). Insect migration is regulated not only by the environmental conditions but also by the insect physiological factors (Riley et al. 1991, Mcneil et al. 2000, Riddiford 2012). Light is an important environmental factor to affect insect migratory behavior (Harrison 1980, Cao et al. 1997). With the development of molecular biology technology, there is increasing study and focus on the genetic and molecular basis of long-distance migration. Many studies have demonstrated that *cryptochromes* act as the molecular components of circadian clock that involved in the regulation of migratory activity in many animals (Zhan et al. 2011, 2014, Li 2016). Furthermore, *cryptochromes* also can act as magnetoreceptors that sensed the directional and positional information of the magnetic field for successful navigation during animals' migration (Mouritsen et al. 2004, Maeda et al. 2012, Yoshii et al. 2012, Dodson et al. 2013). So far, it is unclear how specificity would have arisen with respect to magnetic information and how magnetic information would be distinguished from circadian input. Thus, the mechanism of the coupling of circadian behavior and magnetosensing by the expression of *cry* genes deserves study.

Agrotis segetum Denis and Schiffermaller is commonly known as the turnip moth and causes considerable damage to crops and vegetables in Europe, Asia, and Africa (Lv et al. 2006, Esbjerg and Sigsgaard 2014, Lemic et al. 2016, Nowinszky et al. 2017). Meanwhile, the turnip moth is an important migratory pest and its seasonal migration in China has been studied (Guo et al. 2015, 2016; Chang et al. 2018a). Previous studies have indicated that the *cry* genes might be associated with migratory activity (Zhan et al. 2011, Wan et al. 2014, Xu et al. 2015, Li 2016); we thus speculated that *A. segetum* may also use *cry* genes to regulate the migration behavior and sense the geomagnetic fields to obtain inclination information during their migration. To explore this hypothesis, we cloned the *cry* genes from *A. segetum* and compared their expression levels between a migratory population and a reared population to investigate whether *cryptochromes* associate with migration. Furthermore, light is the most predominant environmental cue to affect circadian rhythms and migratory activity. CRYs act as photoreceptors and integral components of the circadian oscillator protein complex, and we also compared the expression levels of *cry* genes under different photoperiod conditions to investigate whether the expression of *cryptochromes* can be affected by light. Our study provides a molecular basis for further research on functional characterization of CRYs in photoreception and magnetoreception by *A. segetum* during its migration.

Materials and Methods

Insect Rearing
Agrotis segetum moths were captured using searchlight traps at night on the 2.5 km^2 island of Beihuang (38° 24′ N; 120° 55′ E) in the Bohai Strait, where is an immigration during May–June and emigration during August–October pathway for the seasonal migration long-distance movement of many insect species between northeastern region and southern/central/northern regions of China (Feng et al. 2009, Guo et al. 2015, He et al. 2018). The moths collected and bred method were performed as described previously (Chang et al. 2017), with a photoperiod of 14:10 (L:D) h (14L/10D; the lamp was turned on at 06:00 and turned off at 20:00) in larvae and adults.

Total RNA Extraction and cDNA Synthesis
Total RNA was extracted from moths with *TransZol* Up Plus RNA Kit (TransGen Biotech, Beijing, China). The first-strand cDNA was synthesized with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China) according to the manufacturer's instructions and applied to conserved cloning as a template.

Cloning of the Encoding cDNA of *As-cry1* and *As-cry2* Genes
All the primers were designed using Primer Premier 5.0 (Premier Biosoft, CA; Table 1). Polymerase chain reaction (PCR) amplification of conserved nucleotide regions for each gene (*cry1*: Cry1F and Cry1R; *cry2*: Cry2F and Cry2R) were performed on GeneAmp PCR System 9700 machine (Applied Biosystems, Foster City, CA) using the following conditions: 94°C preincubation for 5 min; 94°C for 45 s, 60°C for 45 s, 72°C for 2 min, for 35 cycles; and 72°C final extension for 10 min. PCR products were inserted into the pEASY-T3 vector (TransGen Biotech, Beijing, China) and sequenced by Taihe Biotechnology Company (Beijing, China).

According to the procedures of rapid amplification of cDNA end technique, the full-length cDNAs of *cry* genes were obtained. Briefly, the 5′- and 3′-ends of *cry* genes receptors were amplified using the universal primer mix (BD Biosciences, San Jose, CA) with specific primers (Table 1). PCR thermal cycling conditions were 94°C for 5 min; 35 cycles of 94°C for 30 s, 65°C for 5′-RACE, 65°C for Cry1-3′-RACE, 68°C for Cry2-outer-3′-RACE and 60°C for Cry2-inner-3′-RACE 30 s, and 72°C for 1 min; and 72°C for 10 min. All PCR products were cloned into pEASY-T3 vector and sequenced as detailed already.

Bioinformatic Analysis
The whole cDNA sequence and deduced amino acid sequence were analyzed using DNAMAN (Lynnon Biosoft, San Ramon,

Table 1. Primer sequences used for gene cloning and real-time quantitative PCR

Primer name	Primer sequence (5′–3′)
CRY1F	GGCACGTGTATCGTTTCATGG
CRY1R	CTTCCACATTTCGCGCCTTC
CRY2F	CTTAACTCCCGGCTGTTCGT
CRY2R	TCGATGACGGCGGAATAAGG
CRY1-5′-primer	GGATGCCCCCGTTAGCCTTGATG
CRY1-3′-primer	AGCGCGCATGCCTGTGGAGT
CRY2-5′-primer	CCCCTCCGATCCAAACGGGAGGTT
CRY2-3′ inner-primer	TGCTCAAGGCTATGCTAATAGTCC
CRY2-3′ outer-primer	GCAGCCAAGCACCTGATCAACCGTCG
Cry1-QFP	AGCAAGATTGCGAGCCAGTG
Cry1-QRP	CGCCGATAGTTGTTACCGTGT
Cry2-QFP	AAAGCGCCTCTCACGTACCA
Cry2-QRP	TGGTCATCGGTTACTGGCGT
Actin-F	TCCCTCTCCACCTTCCAACA
Actin-R	ACAAGCGTAATTTGAGCCGC

CA), and the open reading frame (ORF) was identified using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Protein functional domains were performed using the ScanProsite (http://www.expasy.org/prosite) and InterPro program (http://www.ebi.ac.uk/interpro/). Multiple alignment was performed using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). A phylogenetic tree was constructed by MEGA 5.0 using the method of neighbor joining. The statistical significance of the neighbor joining tree topology was evaluated by bootstrap analysis with 1,000 replicates (Kumar et al. 2008).

Real-Time Quantitative PCR Analysis

The mRNA expression of *cry* genes was analyzed by real-time quantitative PCR (qRT-PCR) using SuperReal PreMix Plus (SYBR Green; Tiangen) according to the supplier's instructions. Female or male 3-d-old moths were dipped into a liquid nitrogen container to separate the head, thorax, abdomen, antennae, legs, and wings. We also wanted to know about the relationship between the mRNA expression of developmental stages, photoperiod conditions, and biological function. The developmental expression of *cry* genes was performed by analyzing the samples of larval, pupae, and adults (from 1- to 8-d-old moths, collecting moths every 2 d). To investigate whether the expression of *cry* genes can be affected by light, head RNA was extracted from a pool of newly emerged and synchronized 2-d-old females moths, reared in 14L/10D, LL (constant light), DD (constant darkness) after they emerged, and female moths were collected every 4 h during the next 24 h (one day is divided into 24 h, the 0- and 24-h time points correspond to midnight, as for our local clock time; sampling time point is ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24, respectively).

The primers used for *cry* genes and the control gene *actin* are shown in Table 1. The PCR were performed with 7500 Real-time PCR System (Applied Biosystems) using the following conditions: an initial denaturation step of 15 min at 95°C, followed by 40 cycles at 95°C for 10 s and 60°C for 32 s, and dissociation protocol. The expression level of each target mRNA relative to *actin* mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Expressions of *As-cry1* and *As-cry2* in Migratory Population of *A. segetum*

Migratory *A. segetum* moths were captured on Beihuang Island in 2016 and 2017. A vertical-pointing searchlight trap for sampling migrating insects up to ≈500 m above ground level was placed on a platform ≈8 m above sea level (Feng et al. 2009). Trapping was carried out every night from sunset to sunrise except when there was a heavy rain. There are no arable lands or host crops for *A. segetum*, and daily studies confirmed that no *A. segetum* larvae survived on the island. Therefore, the captured *A. segetum* moths were confirmed to be migrants (Guo et al. 2015).

The number of captured *A. segetum* individuals was recorded every day. We divided the temporal pattern of migratory *A. segetum* captured on Beihuang Island into two periods using Fisher optimal dissection method, namely the immigration period and the emigration period. The immigration and emigration moths were captured from May to July and from September to October, respectively. And each group included 30 samples of migrating *A. segetum*. Total RNA was extracted to investigate the expression patterns of *cry* genes of *A. segetum* migrants, and cDNA was synthesized and processed for qRT-PCR as described already.

Statistical Analyses

The results expressed as the mean ± SD of three parallel measurements. Dates were analyzed using one-way ANOVA, followed by

Turkey's test, and significance was set at $P < 0.05$. Student's *t*-test was used to compare the mRNA expression levels of *cry* genes between female and male moths, between emigration and immigration moths, and between 2016 and 2017. All statistical analyses were conducted using SPSS 20.0 software (SPSS, Chicago, IL).

Results

Molecular Cloning of *As-cry1* and *As-cry2* cDNA

The full-length cDNA of *cry* genes were obtained in *A. segetum*, and they were designed as *As-cry1* and *As-cry2*. The complete nucleotide sequence of *As-cry1* was 1,881 bp, which contained a 5′ untranslated region (5′-UTR) of 102 nucleotides, and an ORF of 1,581 nucleotides encoding 527 amino acids, followed by a 198 nucleotides 3′-UTR; Fig. 1A). The full-length *As-cry2* cDNA contained a 5′-UTR of 369 bp, and ORF of 2,358 bp, and a 3′-UTR of 388 bp and encoded 786 amino acids (Fig. 1B). As shown in other CRYs sequences, domains of DNA photolyase and flavin adenine dinucleotide (FAD) binding 7 were identified in the deduced amino acid sequences of *As-cry1* and *As-cry2* (Fig. 1). These domains were signature characterization in the CRY family (Yan et al. 2013, Xu et al. 2015), appeared highly conserved among the different insects.

Phylogenetic Analysis of *As-cry1* and *As-cry2* mRNA

Multiple alignments showed that the deduced amino acid sequence of As-CRY1 is very similar to the CRY1s from *Agrotis ipsilon* (Lepidoptera: Noctuidae) (96.4%), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (94.5%), *Bombyx mori* (Lepidoptera: Bombycidae) (80.7%), *Danaus plexippus* (Lepidoptera: Danaidae) (80.6%), and *Drosophila melanogaster* (55.7%; Fig. 2A). On the other hand, As-CRY2 showed higher amino acids identity with CRY2s in *A. ipsilon* (94.3%), *H. armigera* (83.3%), *B. mori* (69.7%), *D. plexippus* (66.7%), and *Nilaparvata lugens* (Homoptera: Delphacidae) (49.3%; Fig. 2B). Moreover, the similarity was 27.3% between As-CRY1 and As-CRY2.

Multiple alignments also revealed that the N-terminus (39.5%) of the protein had higher levels of conservation than the C-terminal (15.5%). The highly conserved regions of As-CRY1 and As-CRY2 contained DNA photolyase domains and FAD-binding 7 domains, which were highly conserved among the different CRYs.

According to the phylogenetic analysis, the total 18 CRY proteins were classified into two clusters: insect CRY1 and insect CRY2, and As-CRY1 and As-CRY2 belonged to the CRY1 and CRY2 clusters, respectively (Fig. 3). The phylogenetic tree was similar in topology tree that has been previously reported (Yan et al. 2013). Moreover, the results showed that As-CRYs were closely related to CRYs from Noctuidae species, but relatively distant from CRYs of Delphacidae, Bombycidae, Drosophilidae, Danaidae, and Apidae species.

Relative Expression Abundance of *As-cry1* and *As-cry2* during Developmental Stages

The relative expression abundance of *cry* genes were significantly different from larvae to adult stage (*As-cry1*: $F_{10, 22} = 3,965.55$, $P < 0.001$; *As-cry2*: $F_{10, 22} = 1,786.11$, $P < 0.001$; Fig. 4A and B). The *cry* genes showed low expression levels in the larvae, with a subsequent increase, and the peak expression of *cry* genes occurred on 5- to 6-d-old moths. The expression of *As-cry1* was lower in younger adults, then significantly increased in older adults ($F_{3, 8} = 921.833$, $P < 0.001$). The expression pattern of *As-cry2* was similar to that of *As-cry1* during the adult stage ($F_{3, 8} = 699.795$, $P < 0.001$).
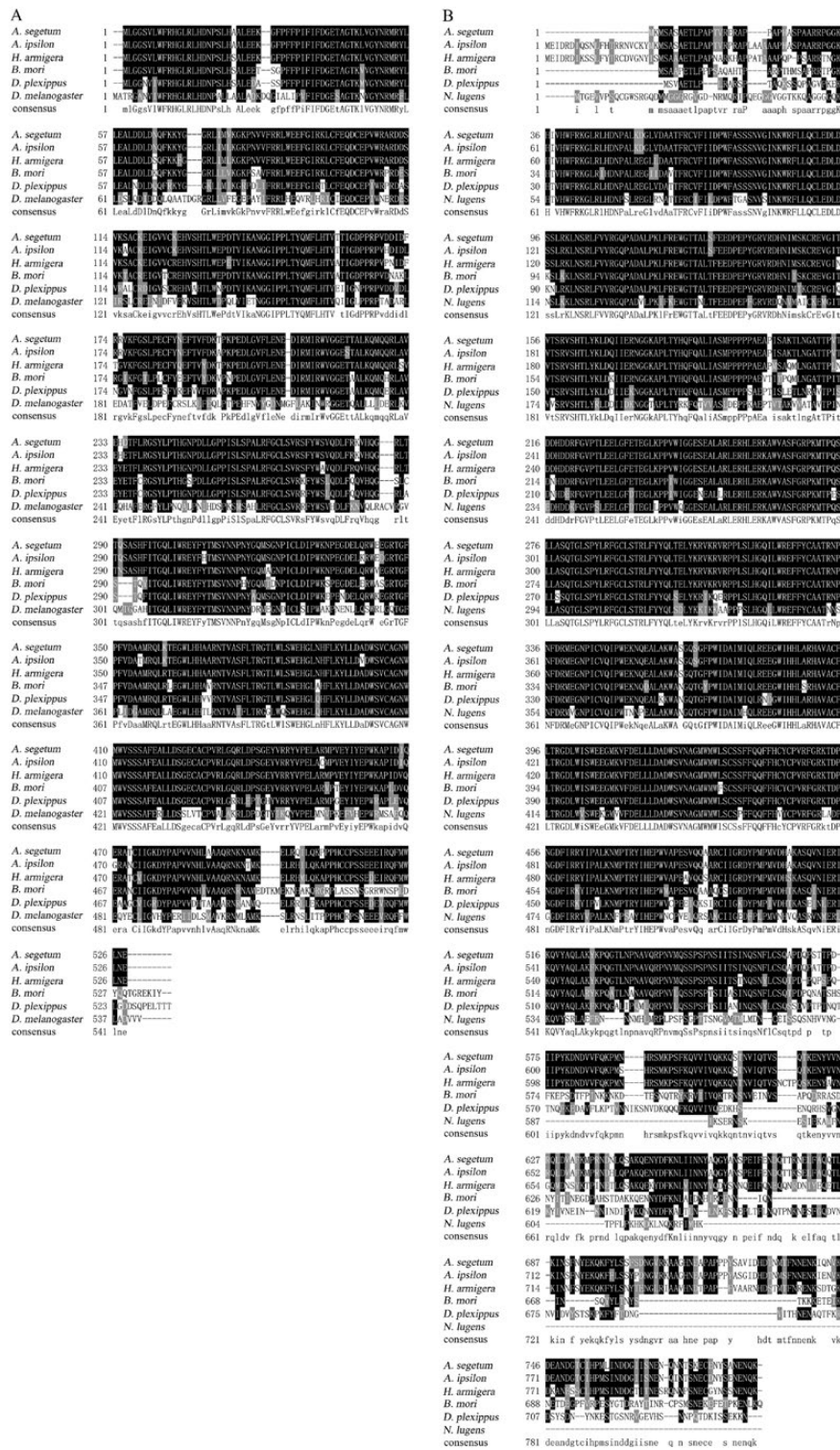
A

B

Fig. 1. Nucleotide sequence and deduced amino acid sequence of complete *As-cry1* (A) and *As-cry2* (B) cDNA. The predicted start ATG and stop TAA codons, and polyadenylation signal are shaded in gray. DNA-photolyase domains are shaded in yellow; flavin adenine dinucleotide (FAD)-binding-7 domains are shaded in blue.

Relative Expression Abundance of *As-cry1* and *As-cry2* in Different Tissues

As-cry1 and *As-cry2* mRNA were present in head, thorax, abdomen, antennae, legs, and wings of adult females and males. The maximal relative expression abundance of *As-cry1* mRNA in both adult females and males was found in the heads, with the minimum relative expression abundance were found in the thorax and abdomen of adult females and males, respectively. *As-cry1* mRNA in the various tissues was significantly different (females: $F_{5,12} = 4.51$, $P < 0.05$; males: $F_{5,12} = 31.897$, $P < 0.001$; Fig. 4C). Similar to the *As-cry1* mRNA expression, the maximal relative expression abundance of *As-cry2* mRNA was also found in the heads of adult

Fig. 2. Multiple alignment of predicted amino acid sequences of CRY1 (A) and CRY2 (B) from *Agrotis segetum* with CRY proteins from other insects. Highly conserved amino acids are shaded in black. (A) GenBank accessions: *Agrotis ipsilon* (JQ616846.1), *Bombyx mori* (NM_001195699.1), *Helicoverpa armigera* (JN997418.1), *Danaus plexippus* (AY860425.1), and *Drosophila melanogaster* (AF099734.1). (B) GenBank accessions: *A. ipsilon* (JQ616847.1), *B. mori* (NM_001195698.1), *H. armigera* (JQ713142.1), *D. plexippus* (DQ184682.1), and *Nilaparvata lugens* (KM108578.1).

females and males. Relative expression abundance of *As-cry2* mRNA in the heads were significantly greater than that in the five other tissues (female: $F_{5,12} = 16.347$, $P < 0.001$; male: $F_{5,12} = 89.105$, $P < 0.001$; Fig. 4D). The relative expression abundance of *As-cry2* in both adult females and males were similar to that of *As-cry1*.

Relative Expression Abundance of *As-cry1* and *As-cry2* under Different Photoperiods

The results showed that the expression abundance of both *cry* genes occurred in a diurnal rhythm in 3-d-old females under 14L/10D condition (Fig. 5). The expression abundance of As-*cry1* was higher in
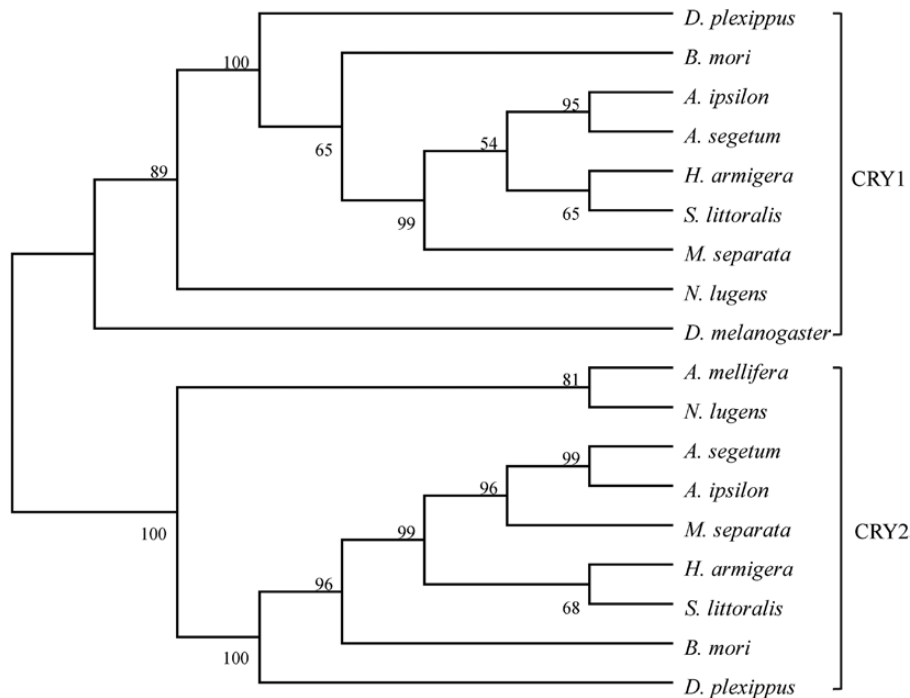
Fig. 3. Phylogenetic analysis based on As-CRY1 and As-CRY2 amino acid sequences using the neighbor-joining method. The numbers at the nodes indicate bootstrap values. GenBank accessions: *Agrotis ipsilon*-CRY1 (JQ616846.1), *Bombyx mori*-CRY1 (NM_001195699.1), *Helicoverpa armigera*-CRY1 (JN997418.1), *Drosophila melanogaster*-CRY1 (AF099734.1), *Nilaparvata lugens*-CRY1 (KM108579.1), *Danaus plexippus*-CRY1 (AY860425.1), *Mythimna separata*-CRY1 (JX077108.1), *Spodoptera littoralis*-CRY1 (EF364035.1), *A. ipsilon*-CRY2 (JQ616847.1), *B. mori*-CRY2 (NM_001195698.1), *H. armigera*-CRY2 (JQ713142.1), *Apis mellifera*-CRY2 (NM_001083630.1), *N. lugens*-CRY2 (KM108578.1), *D. plexippus*-CRY2 (DQ184682.1), *M. separata*-CRY2 (JX077109.1), and *S. littoralis*-CRY2 (EF396286.1).

the day than in the night. In contrast, the expression abundance of As-*cry2* was higher in the night than in the day.

The highest expression levels of As-*cry1* gene in 3-d-old females occurred at ZT8, followed by ZT12, ZT4, ZT20, ZT0, ZT24, and ZT16 in decreasing order in 14L/10D (Fig. 5A). There were significantly different among seven of the time points for the expression levels of As-*cry1* gene in 14L/10D ($F_{6, 14}$ = 207.07, P < 0.001). Similar to the expression levels of As-*cry1* in 14L/10D, the expression of As-*cry1* in DD also significantly different among the different time points (DD: $F_{6, 14}$ = 7.445, P < 0.001). In contrast to the As-*cry1* expression in 14L/10D and DD, there were no significant differences among the different time points for the expression of As-*cry1* (LL: $F_{6, 14}$ = 2.167, P = 0.109). The expression abundance of As-*cry1* during the day in average was higher than expression in the night (the average expression in the day: 2.707, in the night: 1.077; t = −23.936, df = 4, P < 0.001).

For the relative expression abundance of As-*cry2*, there were significantly different among the different time points in 14L/10D, LL, and DD (14L/10D: $F_{6, 14}$ = 9.519, P < 0.001; LL: $F_{6, 14}$ = 21.147, P < 0.001; DD: $F_{6, 14}$ = 7.738, P < 0.001; Fig. 5B). The maximal relative expression abundance of As-*cry2* mRNA were occurred at ZT8, ZT24, and ZT20, with the minimum expression occurred at ZT16, ZT4, and ZT8 in 14L/10D, LL, and DD, respectively. Contrary to the expression level of As-*cry1*, the expression of As-*cry2* during the day in average was lower than expression in the night (the average expression in the day: 0.771, in the night: 0.984; t = 4.365, df = 4, P < 0.05).

Relative Expression Abundance of *As-cry1* and *As-cry2* in Migratory Populations

According to the number of captured *A. segetum* individuals in 2016 and 2017, and the optimal temporal pattern of migratory *A. segetum*

captured on Beihuang Island (Guo et al. 2016), the immigration and emigration groups were separately analyzed in 2016 and 2017 (Fig. 6A and B). RNA was isolated from the immigration and emigration moths, and the expression levels of *cry* genes were analyzed.

The expression of As-*cry1* and As-*cry2* in migratory moths significantly differed between the immigration group and the emigration group (2016, As-*cry1*: t = −13.353, df = 4, P < 0.001; As-*cry2*: t = −30.251, df = 4, P < 0.001; 2017, As-*cry1*: t = −16.965, df = 4, P < 0.001; As-*cry2*: t = −31.099, df = 4, P < 0.001; Fig. 6C and D). The expression levels of As-*cry1* and As-*cry2* were significantly higher in the emigration group than in the immigration group in 2016 and 2017. The expression pattern of *cry* genes in 2016 was similar to that of in 2017. Furthermore, the expression of each of the two *cry* genes differed significantly between 2016 and 2017 in the immigration moths and in the emigration moths (repeated measures, P < 0.001). In addition, the expression levels of As-*cry1* and As-*cry2* in migratory moths were higher (Fig. 6C and D) than in the reared population (repeated measures, P < 0.001; Fig. 4A and B).

Discussion

Many species can sense magnetic fields for the purpose of orientation and/or to navigate and migrate over a long distance (Maeda et al. 2012, Wiltschko and Wiltschko 2015, Worster et al. 2016). Cryptochromes remain the best suitable candidate molecular to be used by migratory birds (Heyers et al. 2007) and insects (Xu et al. 2015) to sense and respond to the direction geomagnetic fields during their navigation. Meanwhile, cryptochromes may be involved in the regulation of migratory activity in many animals and/or even for circadian-rhythm behavior in some species (Zhan et al. 2011, Qin et al. 2015, Li 2016).
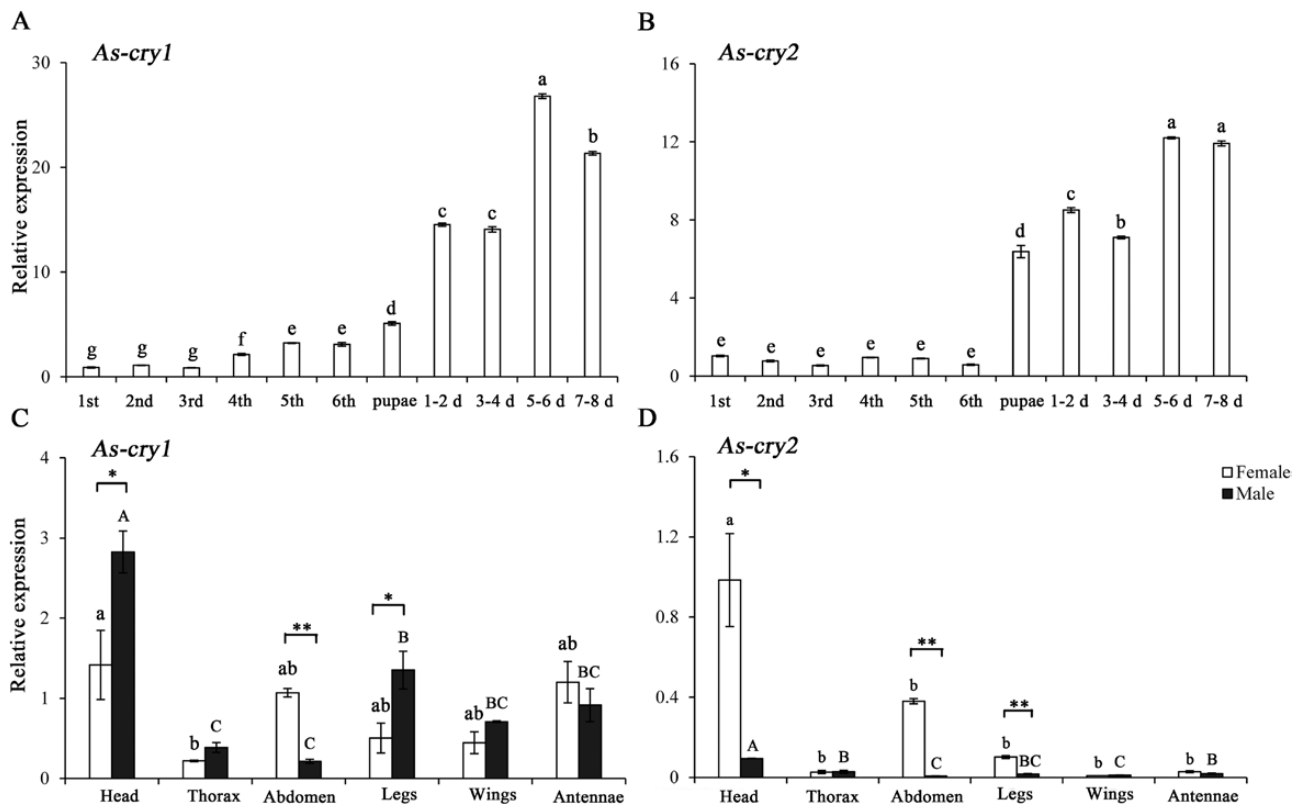
Fig. 4. Relative mRNA levels of *As-cry1* (A) and *As-cry2* (B) during development, and transcript levels of *As-cry1* (C) and *As-cry2* (D) in different organs of 3-d-old male and female of *Agrotis segetum* moths as determined by real-time quantitative PCR (*Actin* as internal standard). Each value is the mean ± SE of three collections. Lowercase letters above the bar indicate significant differences among the developmental stages according to Tukey's honestly significant difference (HSD) tests ($P < 0.05$) in (A) and (B). Lowercase letters above the bar indicate significant differences among females according to Tukey's HSD tests ($P < 0.05$), and capital lowercase letters above the bar indicate significant differences among males according to Tukey's HSD tests ($P < 0.05$) in (C) and (D). Asterisks above bars indicate a significant difference between males and females according to a *t*-test (*$P < 0.05$, **$P < 0.001$).
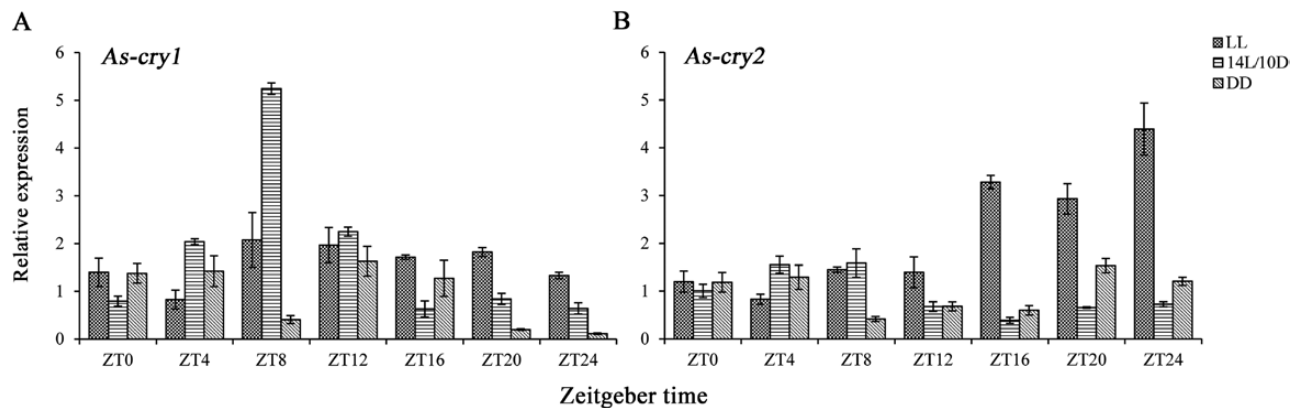

Fig. 5. Relative mRNA transcript levels of *As-cry1* (A) and *As-cry2* (B) as determined by real-time quantitative PCR of DNA from *Agrotis segetum* moths exposed to different photoperiods. *Actin* was used as an internal standard. Heads were collected at 4-h intervals for 24 h. Each value is the mean ± SE of three collections.

In this article, we for the first time succeeded in isolating the full length of *cry1* and *cry2* cDNA sequences from *A. segetum*. Previous researches have showed a basis for the correlation between amino acid domains and the function of CRYs (Sancar 2003, Müller and Carell 2009, Rodgers and Hore 2009, Dodson et al. 2013). Studies have demonstrated that the conserved tryptophan (Trp) residues in the photolyase homology region domain and FAD cofactor were necessary for magnetosensitivity activity of CRY molecules (Rodgers 2009, Mouritsen and Hore 2012, Dodson et al. 2013, Mei and Dvornyk 2015, Muheim and Liedvogel 2015), and the photolyase

homology domain was necessary for light detection and phototransduction (Busza et al. 2004). Our results showed that the DNA photolyase and FAD domains were identified in As-CRYs were highly conserved. The structural conservation of these domains among different species might be evidence of common mechanistic features, particularly in magnetoreception (Qin et al. 2015) and photoreception (Merlin et al. 2006). In contrast, the N- and C-terminal extensions were varied widely between species and class of cryptochromes, and it presumably reflected their different physiological roles (Dodson et al. 2013). The phylogenetic tree showed that
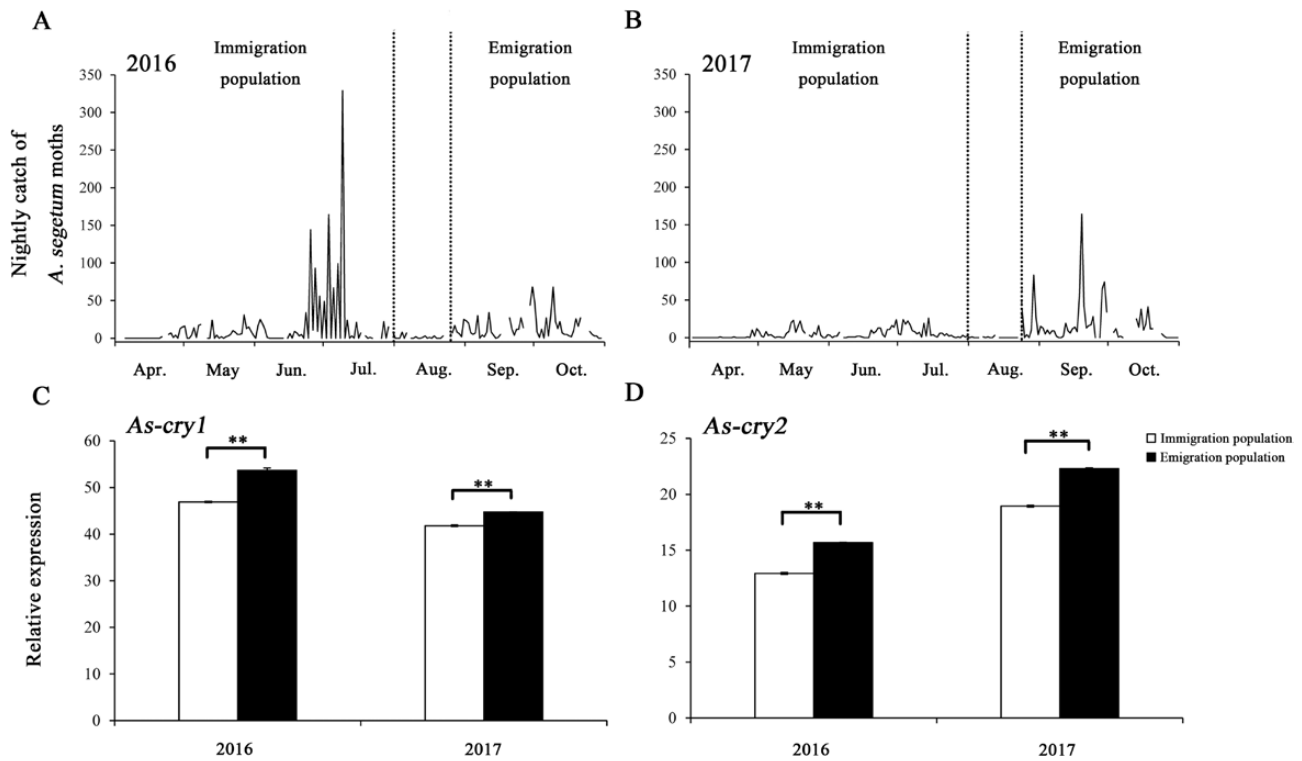
Fig. 6. Nightly catches of *Agrotis segetum* in the searchlight trap on Beihuang Island from April to October in 2016 (A) and 2017 (B). Relative mRNA transcript levels of *As-cry1* (C) and *As-cry2* (D) in the whole body of migratory populations in 2016 and 2017 as determined by real-time quantitative PCR. *Actin* was used as an internal standard. Each value is the mean ± SE of three collections. Asterisks above bars indicate a significant difference between the immigration and the emigration moths according to a *t*-test (**$P < 0.001$).

As-CRY1 and As-CRY2 belong to the CRY1-d and CRY2-m family, respectively. Moreover, the CRYs sequences of *A. segetum* were closer to that of *A. ipsilon* and *H. armigera* than to those of non-lepidopteran insects. This result conformed well to the traditional classes of these species.

Based on previous studies, CRYs are expressed in the eyes and heads of migratory birds (Möller et al. 2004, Mouritsen et al. 2004), where crucial magnetoreceptors have been localized (Prior et al. 2004). However, our qRT-PCR results reveal that *cry* genes were expressed in all test organs of turnip moth. And CRYs are also expressed in the circadian clock neurons of mice and flies (Egan et al. 1999, Maywood et al. 2003, Yoshii et al. 2008, Zheng et al. 2008) and in all test tissues of insects (Yan et al. 2013, Xu et al. 2015, Chang et al. 2017) and animals (Zhou et al. 2016, Wang et al. 2017). Those results raise the possibility that CRYs act as the molecular components of circadian clock that involved in the regulation of migratory activity in *A. segetum*. Furthermore, the result showed that there were sex differences in the expression levels of *cry* genes, similar to the case of *A. ipsilon* adults (Chang et al. 2017). Explanations for this result may be that the function of CRYs is diverse and depends on the insect physiology. CRYs act as photoreceptors in the brain and have other roles elsewhere, such as CRYs act in the entrainment pathway of the clock in the brain, CRYs participate in the circadian rhythm-generating process in peripheral body tissues (Emery et al. 2000; Cashmore 2003; Busza et al. 2004; Merlin et al. 2006, 2007; Agrawal 2016). Besides, reproductive organs exist in the abdomen of insects, and the physiology of females and males differs in the abdomen. So, the different expression levels of *cry* genes in the female and male moths may contribute to differences in behavior and physiology. Here to further research is needed to verify this possibility.

The expression abundance of both *cry* genes was correlated with developmental stages. Our results showed that the expression abundance of both *cry* genes was higher in adults than in larvae and pupae in *A. segetum*. Those findings are in agreement with the results in *A. ipsilon* (Chang et al. 2017). Because *A. segetum* are long-distance migrants (Guo et al. 2015), and As-CRYs may act as magnetoreceptors (Zhu et al. 2008, Nießner et al. 2011, Qin et al. 2015). So, we infer that the high expression level of both *cry* genes in adult stages is necessary to maintain enough cryptochromes proteins and to sense the geomagnetic fields during their migration. Furthermore, As-CRYs act as photoreceptors, which located in compound eyes in Lepidoptera, resulted in relatively higher abundance levels of adults (Chase et al. 1997, Briscoe 2008). The difference in the expression of *As-cry1* and *As-cry2* among the various adult stages may be related to differences in the functions of As-CRY1 and As-CRY2 in *A. segetum*. *As-cry1* belonged to the *cry1-d* family, so the function of CRY1 in *A. segetum* was same as in *D. melanogaster* and it may mainly be sensitive to UV-A/blue light and function primarily as photoreceptors that synchronize circadian clocks (Gegear et al. 2008, Yoshii et al. 2012). However, *As-cry2* belonged to the *cry2-m* family, so the function of CRY2 in *A. segetum* was same as in *A. ipsilon* and garden warblers, and it may be potent repressors of the transcriptional feedback loop of the circadian clock mechanism and participate in magnetosensing (Mouritsen et al. 2004, Chang et al. 2017). The role of cryptochromes in magnetoreception, photic entrainment, and other physiological functions of *A. segetum* needs to be further investigated.

In *A. segetum* moths under 14L/10D condition, the expression of both *cry* genes occurred in a diurnal rhythm in 3-d-old females. The expression patterns of *As-cry1* and *As-cry2* differed from each other,

which adapted to different functions in *A. segetum*. Those results were consistent with the report on *A. ipsilon* (Chang et al. 2017), *H. armigera* (Yan et al. 2013), and *Spodoptera littoralis* (Merlin et al. 2007). It is reasonable that the expression of *As-cry1* mRNA was higher during the day and *As-cry2* mRNA was higher at night because CRY1 as a blue-light photoreceptor regulates circadian clocks during the day (Gegear et al. 2008, Yoshii et al. 2012) and CRY2 as a magnetoreceptor senses the geomagnetic fields (Mouritsen et al. 2004, Chang et al. 2017). Because CRY1 as the light-mediated magnetoreceptor to sense the magnetic field in animals, *cry1* expression was higher at night than during the day in migratory garden warblers (Mouritsen et al. 2004, Fusani et al. 2014). Therefore, we infer from our results that the diurnal expression of *As-cry1* is associated with circadian photoreception, and nocturnal expression of *As-cry2* is associated with magnetosensing in *A. segetum*. Further research is needed to clarify the function of As-CRYs.

Constant light or darkness apparently disturbed the circadian rhythms, which were observed in 14L/10D condition. These results agree with expression levels reported for *D. plexippus* and *H. armigera* in constant light or darkness (Zhu et al. 2008, Yan et al. 2013). A basic characteristic of circadian rhythms is their ability to be synchronized with the environment by light (Hall 2000, Devlin and Kay 2001, Stanewsky 2003). These results suggested that the 24-h pattern of *cry* genes can be reorganized by altered environmental light/dark cycles in circadian clocks (Nagy and Csernus 2007, Mendoza-Viveros et al. 2016). Moreover, the response of *D. melanogaster* to the magnetic field is dependent on the wavelength and intensity of light (Yoshii et al. 2012). Cryptochromes serve as circadian clock core components and magnetoreceptors, so the expression levels of both *cry* genes can be affected by light. Moreover, our result showed that the expression of *As-cry2* showed stronger change in the LL condition. Explanations for this may be that constant light caused circadian arrhythmicity (Stanewsky 2003) and that CRY2 acts in the negative feedback loop of the circadian oscillator (Emery et al. 1998, Krishnan et al. 2001, Gegear et al. 2008, Nießner et al. 2016), the increased expression of *As-cry2* may be to regulate the circadian rhythm. Furthermore, the expressions of *crys* gene were not only induced by exposure to light, and the light-dependent inductions of *crys* gene might be mediated by more complex mechanism that needs further study.

The expression levels of *cry* genes differed significantly between the immigration population and the emigration population. Compared with the reared population, the migratory population had higher *As-cry1* and *As-cry2* expression levels. Expression levels of *cry* genes were also higher in the emigration population than in the immigration. Cryptochromes are associated with sensing of magnetic fields during the migration of birds (Heyers et al. 2007, Fusani et al. 2014) and insects (Xu et al. 2015). We had speculated that if cryptochromes are involved in magnetic orientation and the regulation of migratory activity during *A. segetum* migration, then expression levels of *cry* genes will be higher in the migratory population. Our results were consistent with the deduction. *Cry1* expression levels in the retina at night are also significantly higher in migratory garden warblers that in non-migratory zebra finches (Mouritsen et al. 2004). Previous studies have showed that *A. segetum* annually migrate to the north from central and southern China in the spring and to the south from northeast China in the autumn (Guo et al. 2015, Chang et al. 2018a). The migratory samples of *A. segetum* were collected on the Beihuang Island in the center of the Bohai Strait, where it is close to northeastern China. Immigration insects collected in the spring were near to arrive in their destination, whereas the emigration moths need to make more trips and take longer time to complete their migration and find new habitats (Guo et al. 2015). Thus, emigration moths would need more CRYs proteins to sense magnetic fields for orientation, and the two *cry* genes were significantly upregulated in the emigration population compared with those in the immigration population. The differential expression of the *cry* genes in the two types of migratory populations of *A. segetum* thus suggests that the two *cry* genes characterized in *A. segetum* might be associated with migration, as demonstrated for *N. lugen* (Xu et al. 2015). Certainly, the natural environment is complex and changeable, so further research is necessary to detail the expression of two *cry* genes of *A. segetum* migrants in different conditions.

In summary, we cloned two *cry* genes in *A. segetum* and found a relatively high homology with CRYs from species of Noctuidae. Both *As-cry* genes were expressed in all tested organs of adults, with highest expression in adults. *As-cry1* and *As-cry2* transcripts oscillated in a circadian manner under normal 14L/10D, and light can affect the expression levels of *As-cry* genes. Transcript levels of both genes were also higher in the migratory populations than in the reared population and higher in the emigration population than in the immigration. Furthermore, we isolated a full-length *IscA1* (the homolog of magnetoreceptor protein, named IscA1) cDNA from *A. segetum* and investigated gene expression levels of *IscA1* under different treatments in *A. segetum* (Chang et al. 2018b). The results showed that the expression profiles of *IscA1* were similar to that of *cry* genes in *A. segetum* (Chang et al. 2018b). These findings provided preliminary evidence on the role of As-CRYs in magnetosensing activity during *A. segetum* migration. Further studies are needed to clarify the function of As-CRYs in the relationship between circadian clocks and magnetosensing during *A. segetum* migration.

Acknowledgments

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