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Identification of a transcription factor that functions downstream of corazonin in the control of desert locust gregarious body coloration

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ABSTRACT

Corazonin (Crz) is a neuropeptide that controls phase-dependent body color polyphenism in locusts. The Crz signaling pathway is responsible for the development of gregarious black patterns in nymphs and determination of the morphometric ratio *F/C* (*F* = hind femur length, *C* = maximum head width) in adults. However, little information is available regarding the mediator and effector proteins regulated by Crz. In this study, we identified a novel transcription factor, Loct, which functions downstream of Crz in *Schistocerca gregaria* and *Locusta migratoria*. In *S. gregaria*, we detected a variant of Loct lacking the N-terminal region. Protein–protein interaction assays showed that both the long and short Loct variants formed a complex with themselves. *LOCT* knockdown in gregarious nymphs reduced the intensity of their black patterning, but did not affect *F/C* ratios in adults. *LOCT* was exclusively expressed in the integument of gregarious nymphs, suggesting that Loct is involved in melanin production. In addition, we found that the melanization-associated protein Yellow (*YEL*) and the albino-related takeout protein (*ALTO*) are expressed in the integument and function downstream of Crz. However, Crz injection failed to influence *LOCT*, *YEL*, and *ALTO* expression. Therefore, additional factors probably cooperate with Crz to induce these genes. The gene expression profiles of *YEL* and *ALTO* in *LOCT*-knockdown nymphs suggest that Loct does not directly control the transcription of *YEL* or *ALTO*. In summary, we present a working model of the Crz pathway, which is active in crowded *S. gregaria* nymphs.

1. Introduction

Neuropeptides regulate various metabolic, homeostatic, developmental, reproductive, and behavioral processes in insects (Gäde and Goldsworthy, 2003). Many neuropeptides have been isolated and identified in the desert locust, *Schistocerca gregaria*, and the migratory locust, *Locusta migratoria* (Schoofs et al., 1997). However, the exact roles of most of these peptides remain unknown.

Schistocerca gregaria and *L. migratoria* show density-dependent phase polyphenism. These locusts are solitarious and gregarious at low and high population densities, respectively (Uvarov, 1977, 1966). During the transition between solitarious and gregarious phases, these locusts exhibit behavioral, physiological, and morphological features that are intermediate between the two phases. This interval is called the transient phase (Pener and Simpson, 2009; Pener and Yerushalmi, 1998). Solitarious nymphs assume various body colors depending on the surrounding environment (Faure, 1932; Tanaka et al., 2012). In contrast, gregarious nymphs develop black patterns with a yellow or orange background in *S. gregaria* and a dirty orange background in *L. migratoria*. The morphometric ratio *F/C* (*F* = hind femur length; *C* = maximum head width) is smaller in gregarious than in solitarious adults (Dirsh, 1951; Pener, 1991).

The neuropeptide [His^7]-corazonin (Crz) is known to control body color (Tawfik et al., 1999) and phase-dependent morphological traits including the *F/C* ratio and pronotum shape in the gregarious phase (Tanaka, 2006; Pener and Simpson, 2009). Synthetic Crz injections cause green solitarious (isolation-reared) nymphs to develop the black patterns usually observed in gregarious forms. In *S. gregaria* and *L. migratoria*, exogenous Crz also shifts the adult *F/C* ratio toward values characteristic of gregarious forms even under isolated conditions (Hoste et al., 2002b; Tanaka et al., 2002). In both locust species, RNA interference (RNAi)-mediated knockdown of *CRZ* in gregarious (crowd-reared) nymphs reduces the intensity of their black patterning (Sugahara et al., 2016, 2015b). In addition, *CRZ*-defective *L. migratoria* and Crz receptor gene (*CRZR*)-defective *S. gregaria* mutants fail to develop normal gregarious body coloration (Sugahara et al., 2017a). It has been proposed that other factor(s) might also cause phase-dependent morphometric differences in *L. migratoria* because the *CRZ*-defective *L.*

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migratoria shows a density-dependent shift in its *F/C* ratio (Hoste et al., 2002a; Yerushalmi et al., 2001). Pener (2017) reported an *F/C* ratio shift in *CRZR*-defective *S. gregaria*; however, we failed to detect such a shift for the same strain (Sugahara et al., 2017a).

Corazonin was named for the word corazón, Spanish for heart, as it was originally identified as a cardioacceleratory peptide in the cockroach *Periplaneta americana* (Veenstra, 1989). However, this function has only been observed in *P. americana* and the blood-feeding bug, *Rhodnius prolixus* (Patel et al., 2014; Veenstra, 1991). The presence of Crz can be easily detected using a highly sensitive albino locust assay; nymphs turn darker in response to injected Crz (Tawfik et al., 1999; Hua et al., 2000). Corazonin activity is observed in 18 orders of insects except for Coleoptera, suggesting that Crz may be common in various insects (Tanaka, 2006, 2008). In fact, Crz signaling is widespread among invertebrates (Zandawala et al., 2017). Nevertheless, the function of this pathway varies among species. For example, in the ponerine ant, *Harpegnathos saltator*, Crz plays important roles in caste identity and behavior (Gospocic et al., 2017). These functions may also be conserved in other social insects such as the dampwood termite, *Zootermopsis nevadensis*; the honeybee, *Apis mellifera*; and the red paper wasp, *Polistes canadensis* (Gospocic et al., 2017). Corazonin also participates in the larval–pupal transition and puparization of the oriental fruit fly, *Bactrocera dorsalis* (Hou et al., 2017). In the sphinx moth, *Manduca sexta*, Crz injection accelerates the initiation of ecdysis behavior (Kim et al., 2004). However, little information is available regarding the mediator and effector proteins regulated by Crz.

The present study aimed to elucidate the mechanisms controlling phase-dependent body color polyphenism in locusts. We compared the transcriptomes of *S. gregaria* isolation- and crowd-reared nymphs using RNA-sequencing (RNA-seq) analysis and identified many differentially expressed genes (DEGs). Following RNAi-mediated knockdown of ~200 of these genes in antepenultimate instar nymphs, we identified one gene affecting gregarious locust body coloration. This gene encoded the newly discovered locust corazonin-related transcription factor (Loct). We next analyzed the role of Loct in *S. gregaria* nymphs in detail. The DEGs obtained from above analysis included genes encoding the melanization-associated protein Yellow (*YEL*) and the albino-related takeout (*ALTO*). Yellow is a member of the Yellow gene family and several yellow genes have been identified in many insects (Arakane et al., 2010; Futahashi et al., 2008; Maleszka and Kucharski, 2000). In the commercial silkmoth, *Bombyx mori*, *YEL*-deficient larvae, chocolate mutants, visually resemble albino locust nymphs in color (Futahashi et al., 2008; Sugahara et al., 2017a). We previously reported that the yellow protein of the takeout family gene (YPT) controls yellow body color in *S. gregaria* nymphs (Sugahara and Tanaka, 2018). In this study, we focused on *YEL* and *ALTO* and their relationships with *LOCT*.

2. Materials and methods

2.1. Insects

This study used laboratory colonies of wild-type and albino *S. gregaria* strains. The wild-type Niger strain and the albino strain with the defective Crz receptor gene used here have been previously described (Sugahara et al., 2017a). The L. *migratoria* strain was collected from Minami-Daito Island, Japan (Sugahara et al., 2017b). Solitarious individuals were established from the gregarious line, as previously described (Sugahara et al., 2015b). For RNA-seq analysis of the crowding effects, green solitarious nymphs were obtained by rearing green hatchlings individually, while gregarious nymphs were obtained from gregarious black hatchlings reared in groups of 10 individuals per plastic cup (bottom diameter, 8 cm; lid diameter, 9 cm; height, 2.5 cm) covered with yellow-green paper (Tanaka and Nishide, 2012). For RNA-seq analysis comparing wild-type and albino nymphs, locusts were reared in groups of ~100 individuals per wood frame cage (22 × 39 × 43 cm). Double-stranded RNA-injected locusts were raised in lots of > 25 individuals per wooden frame cage.

2.2. Total RNA preparation for RNA-seq analysis

Total RNA was extracted using ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan) either from the whole bodies or the heads of at least three nymphs. Pooled samples were purified using the SV Total RNA Isolation System (Promega Corp., Madison, WI, USA).

2.3. Differential expression analysis

The preparation of cDNA libraries from total RNA for Illumina (San Diego, CA, USA) HiSeq 2000 sequencing was conducted by Macrogen Japan Corp. (Tokyo, Japan). The resulting RNA-sequencing (RNA-seq) reads were filtered by Trimmomatic (Bolger et al., 2014) and qualified reads were assembled *de novo* using Trinity (v. r20140717) (Grabherr et al., 2011). The RNA-seq data obtained in the present study were deposited with the DNA Data Bank of Japan (DDBJ) under accession number DRA006559. Differentially expressed gene (DEG) analysis between crowd- and isolation-reared solitarious nymphs was performed using iDEGES/DESeq (Sun et al., 2013) with a false discovery rate (FDR) < 0.05. Total RNA of the rescuegrass (*Bromus catharticus*) locust feed was also analyzed so that its reads could be recognized and distinguished from those of *Schistocerca gregaria.* Other DEG comparisons were conducted using the iDEGES/edgeR method (Sun et al., 2013) with an FDR < 0.05.

2.4. Determination and cloning of full-length LOCT, YEL, and ALTO cDNA

The 5′ regions of the *LOCT* and *YEL* sequences were determined by 5′ rapid amplification of cDNA ends (RACE) analyses. The cDNA library was derived from the pronota of five-day-old third-instar nymphs. The primers used for RACE analyses and all other full-length *LOCT*, *YEL*, and *ALTO* cloning are listed in Table S1. Full-length *LOCT* was inserted into the pDONR221 vector (Invitrogen, Carlsbad, CA, USA), as described below. Full-length *YEL* and *ALTO* were cloned into the pENTR11 vector (Invitrogen). Nucleotide sequences were submitted to the DDBJ under the following accession numbers: *LOCT-L*, LC369732; *LOCT-S*, LC369733; *YEL*, LC369734; and *ALTO*, LC369735.

2.5. Domain search

A protein motif search was performed with Pfam (v. 31.0). PSORT II (https://psort.hgc.jp) was used to search for signal sequences.

2.6. Cell culture and transfection

The cultured silkworm cell line BmN4 was maintained at 27 °C in EX-CELL 420 medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France). The cells were seeded at a density of $3 \times 10^4\,cm^{-2}$ and incubated overnight prior to transfection. Expression vectors were transfected into the silkworm cells using FuGENE HD (Promega Corp.) during overnight incubation. The transfection medium was replaced with fresh EX-CELL 420 culture medium and the cells were incubated for three days.

2.7. Insect two-hybrid analysis

The full-length open reading frames (ORFs) of *LOCT-L* and *LOCT-S* and the N-terminal regions of *LOCT-L* (*BTB-L*) and *LOCT-S* (*BTB-S*) were amplified using one of the primer pairs listed in Table S1 and cloned into the pDONR221 vector using the gateway BP reaction. The cDNA fragments were transposed into destination vectors containing the N-terminus GAL4 DNA-binding domain or the p65 activation domain using the gateway LR reaction. The constructs were transfected together with the reporter construct pUAS-Luc into BmN4 cells and the

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