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Research paper

Two types of albino mutants in desert and migratory locusts are caused by gene defects in the same signaling pathway

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ABSTRACT

Albinism is caused by mutations in the genes involved in melanin production. Albino nymphs of *Locusta migratoria* and *Schistocerca gregaria* reared under crowded conditions are uniformly creamy-white in color. However, nothing is known about the molecular mechanisms underlying this phenomenon in locusts. The albino strain of *L. migratoria* is known to lack the dark-color-inducing neuropeptide corazonin (Crz). In this study, we report that this albino strain has a 10-base-pair deletion in the gene *LmCRZ*, which encodes Crz. This mutation was found to cause a frame-shift, resulting in a null mutation in Crz. On the other hand, the albino strain of *S. gregaria* is known to have an intact Crz. This strain was found to possess a single-nucleotide substitution in the middle of the Crz receptor-encoding gene, *SgCRZR*, which caused a nonsense mutation, resulting in a truncated receptor. Silencing of *SgCRZR* in wild-type *S. gregaria* nymphs greatly reduced the area and intensity of their black patterning, suggesting that the functional defect of *SgCRZR* likely causes the albinism. The expression level of *SgCRZR* in the albino *S. gregaria* was comparable to that in the wild type. Unlike the wild type, the albino strain of this locust did not show a phase-dependent shift in a morphometric trait controlled by Crz. From these results, we conclude that the mutations in *LmCRZ* and *SgCRZR* are responsible for the albinism in *L. migratoria* and *S. gregaria*, respectively, indicating that the two types of albinism are caused by different genetic defects in the same Crz signaling pathway.

1. Introduction

Albinism is widespread in the animal kingdom (Faure, 1932; Bechtel and Bechtel, 1981; Negishi et al., 1996; Koga and Hori, 1997; Halls, 2004; Anistoroaei et al., 2008). This has implications for the survival and reproductive success of animals, as pigments function to protect animals from ultra-violet irradiation and adorn them with various colors that aid in sexual behavior, camouflage, and mimicry (Protas and Patel, 2008). However, the underlying mechanism of albinism is not well understood (Kamaraj and Purohit, 2014).

In vertebrates, a defect in an enzyme for melanin biosynthesis is responsible for the complete absence or reduction of melanin pigment in the skin, hair, and eyes (Kamaraj and Purohit, 2014). Albinism is also common in insects, such as the cave-dwelling plant hopper *Oliarus polyphemus,* the silkworm *Bombyx mori,* and the pill-bug *Armadillidium vulgare* (Negishi et al., 1996; Bilandžija et al., 2012; Fujii et al., 2013). In *O. polyphemus*, the albinism is caused by a defect in the first step of the melanin synthesis pathway (Bilandžija et al., 2012). In *B. mori*, two albino strains are known, which have defects in the hydroxylation of tyrosine and/or phenylalanine (Fujii et al., 2013). Body color with reduced pigmentation is also observed in the fruit fly *Drosophila melanogaster*, in which albinism is caused by a deficiency in the Yellow protein required for black melanin production (Biessmann, 1985; Wittkopp et al., 2002). However, the cause for albinism in other insects is poorly understood.

Albino locust mutants have been long known (Faure, 1932). In the migratory locust *Locusta migratoria* and the desert locust *Schistocerca gregaria*, albino individuals have been recorded only from laboratory colonies and their albino phenotypes have been shown to be controlled by a simple Mendelian unit (Hunter-Jones, 1957; Hasegawa and Tanaka, 1994). Unlike in most of the other albino animals studied so far (Protas et al., 2006; Fujii et al., 2013; Kamaraj and Purohit, 2014), the albinism in *L. migratoria* is not caused by defects in any gene encoding a pigment-related enzyme or transporter but by a deficiency of a hormonal factor, now recognized as corazonin (Crz) (Tanaka, 1993; Tanaka and Pener, 1994; Tawfik et al., 1999; Baggerman et al., 2001; Roller et al., 2003). However, albino individuals of *S. gregaria* have an intact Crz and methanol extracts of their brain and corpora cardiaca have been demonstrated to exhibit Crz activity (Schoofs et al., 2000; Yerushalmi et al., 2000). Interestingly, Yerushalmi et al. (2000) observed that

Abbreviations: RNAi, RNA interference; Crz, corazonin; CrzR, corazonin receptor; dsRNA, double-stranded RNA; CPRP, a precursor-related peptide; RACE, rapid amplification of cDNA ends; WT, wild type; alb, albino; bp, base pair; aa, amino acid; semi-qRT-PCR, semi-quantitative reverse transcription-PCR; MMEJ, microhomology-mediated end joining; TM, transmembrane; ORF, open reading frame.

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injection of a large quantity of synthetic corazonin (50 nmol) induced some dark color in albino nymphs, and suggested a possibility that this albinism might be caused by a partial defect in the function of the corazonin receptor protein (CrzR); however, their assumption was not based on any molecular studies.

The locust species show density-dependent phase polyphenism; locusts occurring at low and high population densities are often referred to as being in solitarious and gregarious phases, respectively (Uvarov, 1966, 1977; Pener and Simpson, 2009). The dark body color of the gregarious locusts is induced by Crz. In *L. migratoria* and *S. gregaria* nymphs, injections of synthetic Crz caused cuticular melanization (Tawfik et al., 1999; Tanaka, 2000, 2001), and silencing of a gene *CRZ* in gregarious locusts lightened their body color (Sugahara et al., 2015a, 2016). *CRZ* encodes the corazonin precursor, prepro-Crz, which is composed of a signal peptide, Crz, and a precursor-related peptide (CPRP) (Hansen et al., 2001; Boerjan et al., 2010). After the removal of signal peptide and CPRP, a mature Crz is generated, which is released from the nerve cells into the hemolymph for signal transduction. It has been suggested that the process of release controls Crz signaling in density-dependent phase polyphenism (Tanaka and Pener, 1994; Baggerman et al., 2001; Sugahara et al., 2015a, 2016).

The CrzR protein is a member of the family of G protein-coupled receptors, which are located on the cell surface and traverse the cell membrane seven times (Cazzamali et al., 2002). In *D. melanogaster* and the sphinx moth *Manduca sexta*, Crz directly interacts with CrzR (Cazzamali et al., 2002; Kim et al., 2004). This interaction appears to be specific because, as revealed by bioinformatics analysis, Crz and CrzR have co-evolved in invertebrate species (Hauser and Grimmelikhuijzen, 2014). CrzR has not been characterized in locusts.

In this study, we investigated the causes for the two different types of albinism in *L. migratoria* and *S. gregaria* by sequencing the candidate *LmCRZ* and *SgCRZR* cDNAs from the albino strains, respectively. We also determined whether the albino *S. gregaria* would show a density-dependent shift in a morphometric trait, known to be controlled by Crz in the wild type locust.

2. Materials and methods

2.1. Insects

A Minami-Daito strain of *L. migratoria*, which was used as a wild-type strain, was derived from Minami-Daito Island, Okinawa, Japan (Sugahara et al., 2016). The albino strain of *L. migratoria* was established from albino individuals, which appeared in a laboratory colony originating from Okinawa Main Island (Tanaka, 1993). A Niger strain of *S. gregaria* was established from individuals collected in Niger by Prof. H. J. Ferenz in 2004 (Tanaka and Nishide, 2013) and was used as a wild strain in the present study. The albino strain of *S. gregaria* used was originally established in the UK (Hunter-Jones, 1957); it was obtained from Prof. S. Anderson (Copenhagen University, Denmark) through Prof. A. De Loof (KU Leuven, Belgium), and the colony has since been maintained in Japan. All locust colonies were maintained under crowded conditions as gregarious lines; the details of the colony maintenance and rearing methods have previously been described (Sugahara et al., 2015a, 2016). The experiments in this study were carried out with crowd-reared (gregarious) locusts unless otherwise stated. A solitarious colony of the wild-type *S. gregaria* was established from the crowd-reared (gregarious) line by rearing nymphs and adults in isolation for two generations, except for a brief period of mating. They were individually reared in Petri dishes (diameter, 9 cm; height, 2 cm) during the first instar and then were transferred to plastic cups (bottom diameter, 8 cm; lid diameter, 9 cm; height, 2.5 cm), as previously described (Tanaka and Nishide, 2012). The walls of these cups were covered with green paper so that the locusts were isolated visually as well as physically (Tanaka et al., 2016a, 2016b). The solitarious colony of albino *S. gregaria* was established from the gregarious line by rearing the newly hatched in isolation, as described above.

2.2. RACE analyses for LmCRZ and SgCRZR

Total RNA was extracted from the whole body of third instar nymphs of the wild-type and albino *L. migratoria* and wild-type *S. gregaria*, which had their guts removed beforehand, using ISOGEN (Nippon Gene, Tokyo, Japan) and SV Total RNA Isolation System (Promega, Madison, Wisconsin, USA). Complementary DNAs were synthesized from 1.0 μg total RNA using the Generacer SuperScript III RT module (Invitrogen, Carlsbad, California, USA) and oligo(dT) primer.

The full-length cDNA sequence of *LmCRZ* has previously been determined and deposited in the DNA Data Bank of Japan (DDBJ) (Sugahara et al., 2015a, accession number LC031861). The partial cDNA sequence of *SgCRZR* was identified by searching the *S. gregaria* transcriptome data (the DDBJ sequence read archive, accession number DRA002231). The 5′-end of *LmCRZ* and the 5′- and 3′-ends of *SgCRZR* sequences were amplified by rapid amplification of cDNA ends (RACE) according to the GeneRacer protocol (Invitrogen, Carlsbad, California, USA). The first and nested PCRs were performed using KOD-PLUS DNA polymerase (Toyobo, Osaka, Japan); the primers used in the PCRs are listed in Table S1. The resulting PCR products were subcloned into a pCR-Blunt vector (Invitrogen, Carlsbad, California, USA) and sequenced by dye-terminator cycle sequencing on a DNA sequencer 3130 (Applied Biosystems, California, USA).

2.3. Determination of SgCRZR sequence

Total RNA of wild-type and albino *S. gregaria* was extracted from three pronota of third instar nymphs using ISOGEN and SV Total RNA Isolation System. Complementary DNA was synthesized from 0.5 μg total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California, USA) and oligo(dT) primer. The *SgCRZR* fragment was amplified from the cDNA using the primers listed in Table S1. PCR products were cloned and sequenced using standard methods. At least five clones were sequenced for each sample. The partial sequence of *SgCRZR* has been deposited in DDBJ (accession number LC177796).

2.4. RNAi experiments

The *SgCRZR* cDNA was amplified by PCR using specific primers listed in Table S1, and cloned in the pLit vector, which is a dual T7 promoter-driven expression vector (Sugahara et al., 2014). The resulting plasmid was used as a template to amplify the *SgCRZR* cDNA with T7 promoter sequences at both the termini by PCR using T7 promoter primer (Table S1). *dsCRZR* was generated by *in vitro* transcription using the T7 RiboMAX large-scale RNA production kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. A dsRNA that corresponded to the green fluorescent protein variant Venus (*dsVENUS*) was also prepared as a negative control, as previously described (Sugahara et al., 2015a).

To verify the efficiency of *SgCRZR*-knockdown, newly ecdysed third instar nymphs (day 0) were injected once with 2 μL dsRNA solution ($1\ \mu g \cdot \mu L^{-1}$) between the first and second abdominal segments using a capillary after being immobilized on ice (Sugahara et al., 2015a). The knockdown efficiency was determined three days later with semi-quantitative reverse transcription-PCR (semi-qRT-PCR) using total RNA that was isolated from two heads of dsRNA-injected nymphs. PCR was performed using KOD—FX DNA polymerase (Toyobo, Osaka, Japan) and the primers listed in Table S1. For assessing the involvement of *SgCRZR* in darkening of the body color, it was silenced using 2 μL dsRNA solution ($1\ \mu g \cdot \mu L^{-1}$), which was injected twice in the nymphs at the second and third instars.