Helicoidal Organization of Chitin in the Cuticle of the Migratory Locust Requires the Function of the Chitin Deacetylase2 Enzyme (LmCDA2)*\textsuperscript{3}

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In the three-dimensional extracellular matrix of the insect cuticle, horizontally aligned microfibrils composed of the polysaccharide chitin and associated proteins are stacked either parallel to each other or helicoidally. The underlying molecular mechanisms that implement differential chitin organization are largely unknown. To learn more about cuticle organization, we sought to study the role of chitin deacetylases (CDA) in this process. In the body cuticle of nymphs of the migratory locust \textit{Locusta migratoria}, helicoidal chitin organization is changed to an organization with unidirectional microfibril orientation when \textit{LmCDA2} expression is knocked down by RNA interference. In addition, the \textit{LmCDA2}-deficient cuticle is less compact suggesting that \textit{LmCDA2} is needed for chitin packaging. Animals with reduced \textit{LmCDA2} activity die at molting, underlining that correct chitin organization is essential for survival. Interestingly, we find that \textit{LmCDA2} localizes only to the initially produced chitin microfibrils that constitute the apical site of the chitin stack. Based on our data, we hypothesize that \textit{LmCDA2}-mediated chitin deacetylation at the beginning of chitin production is a decisive reaction that triggers helicoidal rearrangement of subsequently assembled chitin-protein microfibrils.

In the 60s and 70s of the last century, Neville (1) pioneered studies of chitin organization in the cuticle of insects. He elegantly described chitin organization levels by ultrastructural analyses using especially the migratory locust \textit{Locusta migratoria} as a model insect. Chitin, the polymer of N-acetylglucosamine residues, interacts with proteins to form microfibrils that are aligned in parallel constituting horizontal sheets (laminae). These sheets are stacked either helicoidally or with unidirectional microfibril orientation along the vertical axis of the cuticle (2). In the locust tibia, helicoidal and non-helicoidal laminar organization alternate periodically reflecting different mechanisms of chitin production and orientation (2). This organization might have a yet unexplored impact on the mechanical properties of the cuticle. Cuticular pore canals connecting the cell surface with the cuticle surface follow chitin orientation; in helicoidally arranged laminae, they are crescentic (a term used by Neville), but in regions of non-helicoidal laminae they are straight (3, 4). Conceivably, chitin organization depends on cuticle proteins that interact with chitin. In a few recent studies, it was demonstrated that so-called CPR (cuticular protein with a Rebers and Riddiford consensus domain) proteins determine hardness in the elytra of the red flour beetle \textit{Tribolium castaneum} (5, 6).

Neville added another level of complexity to the question of cuticle organization. It had been observed that every fifth or sixth sugar residue in chitin is deacetylated (7). He speculated that chitin and the partly deacetylated form of chitin, called chitosan, might be recognized by different sets of yet unidentified proteins. Chitin deacetylation is not a spontaneous event but requires the activity of chitin deacetylases (CDAs)\textsuperscript{4} that belong to the carbohydrate esterase family 4 (CE-4) (8).

Recent phylogenetic analyses of CDA sequences from four insect species showed that insect CDAs can be classified into five groups based on sequence similarity and domain diversity (9). Group I includes CDA1 and CDA2 that have a chitin-binding peritrophin-A domain (ChBD), a low-density lipoprotein receptor class A domain (LDLa), and a polysaccharide deacetylase-like catalytic domain. Group II is composed of CDA3 that has the same domain composition as members of group I CDAs. The overall sequence similarity is, however, as low as 38%. Groups III and IV include CDA4 and CDA5, respectively, which along with the deacetylase signature have a ChBD but no

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\footnotesize4 The abbreviations used are: CDA, chitin deacetylase; ChBD, chitin-binding peritrophin-A domain; LDLa, lipoprotein receptor class A; Serp, Serpentine; Verm, Vermiform; TEM, transmission electron microscope; qPCR, quantitative PCR; dsRNA, double-stranded RNA.
RESULTS

Identification and Characterization of LmCDA2—We identified LmCDA2 coding transcripts from the L. migratoria transcriptome database. Based on sequence analyses, we found two LmCDA2 alternative splicing variants. Comparing the cDNA sequences with the genomic sequence, nine exons spanning a 2.9-kb fragment were recognized. The alternative splicing variants were both composed of eight exons. The alternatively spliced exons were the third and fourth exons that we named exon 3a and 3b, respectively (Fig. 1A). The open reading frame of LmCDA2a and LmCDA2b was 1623 and 1605 bp encoding a protein of 541 and 535 amino acids, respectively. Both proteins, named LmCDA2a (KR537804) and LmCDA2b (KR537805), contain a signal peptide, a ChBD domain, an LDLa domain, and a chitin deacetylase (CDA) domain (Fig. 1B), which is similar to C. T. castaneum (15). Phylogenetic analyses using full-length sequences revealed that LmCDA2a and LmCDA2b belong to insect group I CDAs (Fig. 1C). Additionally, to avoid masking effects of identical sequences in the alternative splicing CDA2 variants, we constructed a phylogenetic tree using only the sequences encoded by exon 3a/b, both of which code for a ChBD motif, and respective sequences in CDA2s of other insect species. This sequence comparison indicated that in all cases the CDA2a and CDA2b isoforms collectively grouped in two separate branches, suggesting early divergence of two different CDA2 variants in insects (Fig. 1D).

Tissue-specific and Developmental Expression Analysis of LmCDA2—To determine the tissue specificity of LmCDA2 expression, seven different tissues, including integument, foregut, midgut, hindgut, gastric caeca, Malpighian tubules, and fat body, were dissected from day 6 of the fifth-instar nymphs, and mRNA expression was examined by using RT-qPCR. The results of RT-qPCR showed that both LmCDA2a and LmCDA2b have the highest expression levels in the foregut, although their expression is low in the other tissues tested (Fig. 2, A and C). To explore the stage-dependent expression pattern of LmCDA2, mRNA expression levels in the integument at days 1–7 of fifth-instar nymphs were monitored by RT-qPCR. The expression of LmCDA2a and LmCDA2b was highest at the first 2 days and lower in the remaining days from day 3 to 7 (Fig. 2, B and D). In summary, the LmCDA2 transcripts were expressed in chitin-producing tissues, and the expression was dynamic underscoring the necessity for tight regulation of chitin modification during the development of the insect.

LmCDA2 Is Required for Locust Molting and Development—To investigate the function of LmCDA2, LmCDA2a, and LmCDA2b at molting, we sought to suppress their expression by injection of dsRNA against LmCDA2 into 2-day-old fifth-instar nymphs. The expression profiles of target genes were examined at 24 h after dsRNA injection by RT-qPCR. Double-stranded GFP (dsGFP) was used as control. The results showed that the expression of LmCDA2a and LmCDA2b were substantially down-regulated in the respective animals with a silencing efficiency higher than 95% compared with transcript levels in dsGFP-injected insects (Fig. 3A). The dsGFP-injected nymphs could molt to adults successfully (Fig. 3B, panel a), whereas the dsLmCDA2-injected nymphs failed to shed the old cuticle and were trapped within the exuviae until they died. Their mortality was 80% (Fig. 3B, panel d). This phenotype was provoked also by injecting dsRNA against the splicing variant LmCDA2a, with a mortality of 85% (Fig. 3B, panel b). However, injection of dsRNA against the splicing variant LmCDA2b had no effect on nymph development, nymph-adult molting, and viability (Fig. 3B, panel c). Injection of dsLmCDA2 into 2nd instar nymphs caused a similar phenotype (supplemental Fig. 5). These results
Chitin Organization Requires Localized CDA2 Activity

A

B

C

D

Exon (100bp) - Intron (1000bp)

Intron >4500bp

ChBD

LDLa

CDA

Group V

Group I

Group II

Group III

OcCDA2b

LmCDA2a

CfCDA2b

BmCDA2b

TcCDA2b

DmCDA2b

TcCDA2a

BmCDA2a

OcCDA2a

CfCDA2a

TcCDA2a

BmCDA2a

OcCDA2a

CfCDA2a
Chitin Organization Requires Localized CDA2 Activity

LmCDA2 Is Needed for Tracheal Cuticle Formation and Feeding—To further evaluate the role of LmCDA2 in cuticle formation, we analyzed the tracheal and the foregut cuticle that are produced by epithelial cells. In dsLmCDA2-injected insects, the taenidiae, which constitute the spiral cuticle of the tracheal system running perpendicular to the length of tracheal tubes, lost their regular spacing and shape. Moreover, their cuticular layers are, in contrast to the plain layers in untreated animals, uneven (Fig. 5A). In particular, the regular shape of the chitinosous procuticle appeared to be deformed. By contrast, we were unable to discern any defect in the foregut cuticle in dsLmCDA2-treated nymphs. This signal was visible at the apical site of the procuticle, but only a weak signal was detected in the dsLmCDA2-injected nymphs (Fig. 4A). Taken together, we conclude that dsLmCDA2 injection not only suppressed LmCDA2 transcript levels but consequently reduced LmCDA2 protein accumulation (Fig. 4A and supplemental Fig. 4). We also conclude that LmCDA2 localizes to the apical site of the procuticle (Fig. 4A).

LmCDA2 Is Needed for Chitin Organization during Cuticle Differentiation—To address why locusts failed to molt after dsLmCDA2 injection, we analyzed the ultrastructure of the nymphal new cuticle with reduced LmCDA2 expression by transmission electron microscopy (TEM). In the nymphs injected with dsGFP, as traced by the crescentic orientation of the pore canals, the procuticle of the body cuticle harbored helicoidally arranged chitin laminae that according to Bouligand (25) appear as alternating electron-dense and electron-lucid horizontal stripes (Fig. 4B). In the dsLmCDA2-injected nymphs, the procuticle had lost its laminar organization. Some faint striations close to the epidermal cells were occasionally visible, however. These probably reflected incomplete down-regulation of LmCDA2 expression. In addition, as judged by the straight pore canals, these laminae were not stacked helicoidally. Furthermore, the procuticle of the dsLmCDA2-injected insects was thicker than the procuticle of the dsGFP-injected insects (Fig. 4, B and C), although the epicuticle was normal.

Chitin Amount Is Unchanged in dsLmCDA2-treated Nymphs—To determine whether the thicker procuticle of dsLmCDA2-injected nymphs is due to increased chitin content, we compared the chitin amounts in dsGFP- and dsLmCDA2-injected insects. Clearly, there were no significant differences in chitin content between the two treatments (Fig. 4D). Thus, increased thickness of procuticle does not correlate with the chitin content.

FIGURE 2. Expression patterns of LmCDA2a and LmCDA2b. A and C, expression of LmCDA2a and LmCDA2b in different tissues on day 6 of 5th-instar nymphs detected by RT-qPCR. B and D, expression of LmCDA2a and LmCDA2b in integuments of fifth-instar nymphs at different days. NSD1 to NSD7, days 1–7 of fifth-instar nymphs. Data are reported as means ± S.E. of three independent biological replicates. Statistical significance was calculated by the Tukey’s test of one-way analyses of variance. Different letters (a–c) above the bars represent groups of significant difference (n = 3) (p < 0.05) among the different tissues and days of fifth-instar nymphs. Data, a, b, and c mean that a is significantly different from b and c, respectively, and that b is significantly different from c.

indicate that LmCDA2a plays a vital role during L. migratoria development and molting.

LmCDA2 Localizes to the Apical Site of the Procuticle—To scrutinize the biological role of LmCDA2, we sought to analyze its localization by immunofluorescence study of the integument. We first showed that the Drosophila CDA2 (Verm)-specific antibody recognizes LmCDA2 by mass spectrometry and Western blotting analyses (supplemental Figs. 1–4 and supplemental Table 1, see also “Experimental Procedures”). Then, we used this antibody for immunodetection experiments. A positive signal was found in both old and newly formed cuticles of the dsGFP-injected nymphs. This signal was visible at the apical site of the procuticle, but only a weak signal was detected in the dsLmCDA2-injected nymphs (Fig. 4A). Together, we conclude that dsLmCDA2 injection not only suppressed LmCDA2 transcript levels but consequently reduced LmCDA2 protein accumulation (Fig. 4A and supplemental Fig. 4). We also conclude that LmCDA2 localizes to the apical site of the procuticle (Fig. 4A).

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FIGURE 1. Gene structure and the phylogenetic analyses. A, exon-intron organization of LmCDA2. Black boxes and lines represent common exons and introns. Red and blue solid boxes and lines indicate two alternative exons, 3a and 3b, and the respective introns. B, alignment of deduced amino acid sequences of TcCDA1, TcCDA2a, and TcCDA2b (T. castaneum) and LmCDA2a and LmCDA2b (L. migratoria). Predicted signal peptide residues are shaded in gray. The ChBD is boxed in purple. The blue shading highlights the conserved six cysteines in ChBD. Note that the spacing and amino acid composition between cysteines 1 and 4 (67–84 amino acids) and the sequence between cysteines 4 and 6 (84–106 amino acids) differ between LmCDA2a and LmCDA2b. These differences might have an influence on chitin-binding efficiency and by consequence enzyme processivity. The green box indicates the low-density LDLa domain. The CDA is underlined in orange. The regions shaded in yellow indicate five conserved motifs, which are essential for the catalytic activity. The black region shows conserved amino acids. The residues coded by alternative exons are highlighted in red. C, phylogenetic analysis of homologous CDA2s from different insect species using the neighbor-joining method of the MEGA version 5 software. Protein sequences are encoded by whole full-length cDNA sequences. The five groups are represented in different colors. D, phylogenetic tree was constructed using the alternatively spliced exon 3 of insect CDA2s applying the neighbor-joining method of MEGA version 5. LmCDA2a and LmCDA2b are marked with blue dots. The brackets embrace the two types of alternatively spliced variants. GenBank Accession numbers are listed in Table 2.
their control counterparts (Fig. 5C). Thus, LmCDA2 plays an important role in feeding, probably due to foregut malfunction, but it apparently is not needed for foregut procuticle organization.

**Discussion**

The insect cuticle is a complex apical extracellular matrix formed by the underlying epidermal cells (37). The main component of the cuticle is the polysaccharide chitin that adopts an ordered organization to contribute to the physical properties of the cuticle. Despite some considerable amount of data, the molecular mechanisms of chitin organization are not well understood. Here, we show that the chitin deacetylase LmCDA2 in *L. migratoria* is indispensable for chitin organization in the body cuticle and the tracheal system but not in the foregut.

*LmCDA2 Function Is Essential in the Migratory Locust—Injection of dsRNA against *LmCDA2* into *L. migratoria* nymphs arrests fifth-instar eclosure. The respective insects are unable to molt and eventually die. Thus, *LmCDA2* is responsible for the nymph-adult molts. Similarly, in *D. melanogaster*, mutations in the *verm* gene coding for a CDA2-type enzyme are embryonic lethal. *D. melanogaster* mutant embryos for both *verm* and *serp* (coding for CDA1) display a stronger phenotype than either single mutant embryo (16). This indicates a partially redundant function of CDA1- and CDA2-type chitin deacetylases in *D. melanogaster*. To what extent
FIGURE 4. Localization of LmCDA2 in the locust integument and effects of dsLmCDA2 injection on locust cuticular structure. A, immunofluorescence detection of LmCDA2 using a polyclonal antibody against CDA2 (Verm) of the fruit fly D. melanogaster on cross-sections of locust abdominal cuticle by confocal laser-scanning microscopy. A secondary Cy3-Affinipure donkey anti-rabbit antibody was applied to detect the anti-Verm antibody (red). Sections were counterstained with FB28 to visualize chitin in the procuticle (blue signal). In dsGFP-injected locusts, LmCDA2 protein (red) localizes apical to the chitin signal (blue). Some red signal is visible in the epidermal cells, probably representing CDA2-containing vesicles. In dsLmCDA2-injected animals, only traces of LmCDA2 protein can be detected. The preimmune rabbit serum was used as negative control. Please note that in our experience the folding of the skin is random and therefore different between the specimens. Epi, epicuticle; Pro, procuticle; oc, old cuticle; nc, new cuticle; es, exuvial space. Moreover, because of resolution limits, the procuticle and epicuticle of the new cuticle cannot be assigned. Scale bar, 20 μm. B, ultrastructure observation by TEM. The cuticle structure was observed after dsLmCDA2 injection using TEM. Boxes in the upper panels indicate the region magnified in the lower panels. The orange brace indicates procuticle thickness. The black arrows indicate the chitin lamina. The chitin laminar organization seen in dsGFP-treated animals (dotted lines delimit laminae) was absent after injection of dsLmCDA2. Some faint striation (*) especially at the basal site of the cuticle close to the epidermal cell can be discerned in these animals. pc, pore canal. Epi, epicuticle; Pro, procuticle. Scale bar, 5 μm and 500 nm. C, thickness of the locust cuticle. The new procuticle between the lower border of the epicuticle and the upper surface of the epidermal cells (orange brace in B) was measured using a ruler after dsGFP or dsLmCDA2 injection. Data were reported as means ± S.E. of five independent biological replications. Statistical significance was analyzed with Student’s t test. *** p < 0.001. D, chitin content analyses of abdominal cuticles of dsGFP- or dsLmCDA2-injected nymphs. Data are presented as means ± S.E. of 10 independent biological replicates. Amounts are given as milligram/g cuticle sample (mg/g). Statistical significance was analyzed with Student’s t test. There is no significant difference between chitin amounts in dsGFP- or dsLmCDA2-injected animals.
LmCDA1 and LmCDA2 have redundant functions remains to be elucidated.

Redundancy among chitin deacetylases is apparently common in insects. Our data indicate that the isoform LmCDA2a is essential, whereas activity of the isoform LmCDA2b seems to be dispensable for survival, although the patterns of LmCD2a and LmCD2b RNA accumulation are similar. Therefore, one may speculate that LmCDA2a (or any other chitin deacetylase) is able to replace LmCDA2b, although LmCDA2b is unable to rescue LmCDA2a suppression. A different complex situation involving chitin deacetylase isoforms is encountered in the red flour beetle T. castaneum. Despite similar expression patterns of the alternative spliced transcripts of TcCDA2, TcCDA2a, and TcCDA2b, the respective enzymes seem to have distinct functions. TcCDA2a is needed for the establishment of the soft femoral-tibial joint cuticle, and TcCDA2b is involved in the formation of the hard elytra (15). Overall, these functional differences are surprising, because the respective isoforms differ only in their chitin-binding domain. The chitin-binding efficiencies of some chitinases may actually depend on differences in their
Chitinous and cuticular defects. Indeed, in the honeybee Apis mellifera where the genes encoding CDA1 and CDA2 are expressed at different stages of development (27). In L. migratoria, some cuticle types such as the foregut and the hindgut are not fully disrupted upon dsLmCDA2 injection. If chitin organization in these tissues requires non-redundant deacetylases are active in them. Taken together, to assess the full impact of chitin deacetylases in L. migratoria, identification and characterization of all LmCDAs are needed.

LmCDA2 Is Necessary to Establish Chitin Organization—Consistent with the data in D. melanogaster and T. castaneum, lethality of LmCDA2 down-regulation is correlated with tra-canal chitin arrangement is lost when LmCDA2 expression is knocked down by RNAi in L. migratoria. This phenotype is also observed in the verm and serp mutant D. melanogaster larval cuticle (18). Instead, chitin microfibrils along the vertical axis of the cuticle, as judged by the straight pore canals, are oriented in parallel. This change in chitin architecture is accompanied by a loss of procuticle compactness.

These findings allow two fundamental conclusions. First, helicoidal packaging of chitin laminae is tighter than non-helicoidal packaging. Second, helicoidal arrangement of chitin laminae requires chitin deacetylation by LmCDA2. The aberrant chitin architecture may explain the lethality of LmCDA2 knocked down insects. Forces needed for eclosion may require mechanical properties of the cuticle conferred by helicoidally and non-helicoidally stacked chitin laminae in the locust tibia as observed by Neville (1) possibly depends on the circadian expression and deposition of LmCDA2 into the growing cuticle. Understanding the molecular mechanisms of cuticle construction in general and chitin organization in particular will potentially have a double impact on molecular agricultural sciences seeking to optimize insect pest control and on material sciences that are inspired by biological materials such as the insect cuticle.

**Experimental Procedures**

**Insects**—The egg masses of L. migratoria were incubated at 28 ± 1 °C, 50% relative humidity, and with a 14-h light/10-h dark photoperiod in the laboratory. After 10 days, the nymphs were reared on fresh wheat sprouts under the same conditions (28). The wheat bran was supplemented after the nymphs reached third instar. Fifth-instar nymphs were used in this study.

**Validation of cDNAs Putatively Encoding Chitin Deacetylases**—Putative chitin deacetylase cDNA sequences were retrieved from the L. migratoria transcriptome by using bioinformatics methods (23). Total RNA was extracted from the integument of fifth-instar nymphs by using RNAisoTM Plus (TaKaRa, Japan). The first-strand cDNA was synthesized from 1 μg of total RNA by using Moloney murine leukemia virus reverse transcriptase (TaKaRa, Japan) and oligo(dT)18 primer (Thermo Fisher Scientific). PCR was performed with the cDNA and a pair of gene-specific primers (Table 1). After specific fragments were purified with the gel purification kit (Omega), each purified product was ligated into the pEASY-T3 vector (Trans-Gen, China). The recombinant plasmid was used to transform Escherichia coli, and positive colonies were identified by bacterial colony PCR. The cDNAs were sequenced in both directions by the Thermo Fisher Scientific.

**Bioinformatic Analysis**—Conceptual translation of cDNA sequences was carried out with translation tools at ExPaSy. Alignments of deduced amino acid sequences of LmCDA2 were conducted with GeneDoc software. Signal peptide was pre-
dicted using SignalP version 4.1 software. Analyses of conserved regions, including the chitin-binding/peritrophin-A domain (ChBD), alow-density lipoprotein receptor class A domain (LDLa), and polysaccharide deacetylase-like catalytic domain (CDA), were conducted with BlastP tools at the NCBI website. Multiple alignments of sequences were conducted by using ClustalX version 1.81 software. MEGA version 5.0 was utilized to construct the phylogenetic tree with the neighbor-joining method (29). Bootstrap analysis was performed by 1000 replications at the cutoff of 50% similarity. The name of genes and the accession numbers are listed in Table 2.

The gene structure was determined by searching the L. migratoria genome with LmCDA2 cDNA sequences as queries by using the NCBI Blast tool. Exons and introns were determined by comparing the genomic DNA and cDNA sequences, and the GT-AG rule was applied to determine the exon-intron boundaries. The exon-intron organization was graphed using the Adobe Illustrator CS3 software (Adobe).

FIGURE 6. Model illustrating the biological role of LmCDA2 in L. migratoria. A, helicoidal organization of chitin microfibrils in the locust cuticle relies on a sequence of molecular interactions starting with the localization of LmCDA2 between the epicuticle and the procuticle that contain lipids and chitin, respectively. Association with lipids and chitin is mediated by the LDLa (blue box) and the chitin-binding domains (green box), respectively. At the beginning, LmCDA2 deacetylates newly produced chitin through its CDA domain (brown box). How many chitin fibers along the vertical axis of the growing procuticle are modified is unclear. B, this partially deacetylated chitin serves as a template for the subsequent helicoidal arrangement of chitin laminae. The pore canals follow this organization and appear as crescentic structures. When LmCDA2 protein is absent, chitin laminae became disturbed and subsequently affect the structure of pore canals.

TABLE 1
Primerss for PCR amplification and dsRNA synthesis

| Application of primers | Gene         | Primer sequence (5’-3’) | Product (bp) |
|------------------------|--------------|-------------------------|--------------|
| cDNA cloning           | LmCDA2a      | F, GGTGAAAGTGAAGCGGTCCA | 1806         |
|                        |              | R, TCCTGGTTGATTTTGGGACTGG |              |
|                        | LmCDA2b      | F, GGTGAAAGTGAAGCGGTCCA | 1788         |
|                        |              | R, TCCTGGTTGATTTTGGGACTGG |              |
| mRNA expression        | β-actin      | F, CGAAGCAAGTCAGAGAGATCT | 156          |
|                        | LmCDA2a      | F, GGTGAAAGTGAAGCGGTCCA | 148          |
|                        |              | R, CATTGCCACAGGACAGTCTTG |              |
|                        | LmCDA2b      | F, CGAAGCAAGTCAGAGAGATCT | 108          |
| dsRNA synthesis        | GFP          | F, taatacgactctatagggGTGGAGGAGGCTTTGG | 571          |
|                        | LmCDA2a      | F, taatacgactctatagggGTGGAGGAGGCTTTGG | 496          |
|                        | LmCDA2b      | F, taatacgactctatagggGTGGAGGAGGCTTTGG | 136          |
|                        | LmCDA2a      | F, taatacgactctatagggGTGGAGGAGGCTTTGG | 108          |
### Table 2
Name of gene and accession number for phylogenetic analysis from NCBI

| Homologous gene | Insects | Accession number |
|-----------------|---------|-----------------|
| DmCDA1          | D. melanogaster | NP730444 |
| DmCDA2          | D. melanogaster | NP730442 |
| DmCDA2a         | D. melanogaster | NP730441 |
| DmCDA2b         | D. melanogaster | NM166810 |
| DmCDA3          | D. melanogaster | NP69806 |
| DmCDA4          | D. melanogaster | NP728468 |
| DmCDA5a         | D. melanogaster | AA5F1567 |
| DmCDA5b         | D. melanogaster | AA5F1568 |
| DmCDA5e         | D. melanogaster | ARU1281 |
| DmCDA5f         | D. melanogaster | AFH03482 |
| DmCDA5g         | D. melanogaster | AFH03483 |
| DmCDA5h         | D. melanogaster | AFH03484 |
| DmCDA5i         | D. melanogaster | AVB53594 |
| DmCDA9a         | D. melanogaster | NP611192 |
| DmCDA9b         | D. melanogaster | NP00126519 |
| TcCDA1          | T. castaneum | NP001095946 |
| TcCDA2a         | T. castaneum | NP001096047 |
| TcCDA2b         | T. castaneum | NP001116303 |
| TcCDA3          | T. castaneum | NP001103905 |
| TcCDA4          | T. castaneum | NP001103906 |
| TcCDA5a         | T. castaneum | NP001103739 |
| TcCDA5b         | T. castaneum | NP001107799 |
| TcCDA6          | T. castaneum | NP001103905 |
| TcCDA7          | T. castaneum | ABW74150 |
| TcCDA8          | T. castaneum | NP001103906 |
| TcCDA9          | T. castaneum | NP001103904 |
| AgCDA1          | Anopheles gambiæ | XP320957 |
| AgCDA2          | A. gambiæ | XP320956 |
| AgCDA3          | A. gambiæ | EJH28929 |
| OcCDA1          | Oxya chinensis | KP271171 |
| OcCDA2a         | O. chinensis | KR537801 |
| OcCDA2b         | O. chinensis | KR537802 |
| CjCDA2          | C. fumiferana | KC295591 |
| CjCDA2a         | C. fumiferana | KC295540 |
| CjCDA2b         | C. fumiferana | KC295591 |
| BmCDA2          | Bombyx mori | NP001103796 |
| BmCDA2a         | B. mori | NP001103795 |
| BmCDA2b         | B. mori | NP001103796 |
| LmCDA2a         | L. migratoria | KR537804 |
| LmCDA2b         | L. migratoria | KR537805 |
| OcCDA1          | Oxya chinensis | NP611192 |
| OcCDA2a         | O. chinensis | KR537801 |
| OcCDA2b         | O. chinensis | KR537802 |
| CjCDA2          | C. fumiferana | KC295591 |
| CjCDA2a         | C. fumiferana | KC295540 |
| CjCDA2b         | C. fumiferana | KC295591 |
| BmCDA2          | Bombyx mori | NP001103796 |
| BmCDA2a         | B. mori | NP001103795 |
| BmCDA2b         | B. mori | NP001103796 |
| LmCDA2a         | L. migratoria | KR537804 |
| LmCDA2b         | L. migratoria | KR537805 |

Tissue-specific and Developmental Expression Analysis of LmCDA2s—Total RNA was extracted using RNAiso™ Plus (TaKaRa, Japan) from each of seven different tissues, including the integument, foregut, midgut, gastric caeca, hindgut, Malpighian tubules, and fat body, dissected from fifth-instar nymphs. A total of 45 nymphs were collected and grouped into three biological replications. The quantity and quality of total RNA were evaluated on 1% agarose gel and NanoDrop 2000 (Thermo Scientific). Two micrograms of PCR product was used as template to synthesize dsLmCDA2 and dsGFP using T7 RiboMAX™ Express RNAi System (Promega) (32). The synthesized dsLmCDA2 and dsGFP were dissolved in the appropriate volumes of deionized water, and the concentration was determined and adjusted to 2.5 μg/μl. Integrity of dsLmCDA2 and dsGFP were confirmed using 1% agarose gel analysis.

The 2nd-day-old fifth-instar nymphs were randomly divided into four groups (each with 40 nymphs) for injection of each dsRNA sample. All qubits of 2.5 μl (6.25 μg) of dsLmCDA2, dsLmCDA2a, dsLmCDA2b, or dsGFP were injected into the hemocoeal between the second and third abdominal segments by using a microinjector (Ningbo, China). To determine silencing efficiency, relative transcript levels of LmCDA2, LmCDA2a, and LmCDA2b were measured at 24 h after the injection of dsRNA by RT-qPCR as described above. Three biological and two technical replicates were applied for dsGFP, dsLmCDA2, dsLmCDA2a, and dsLmCDA2b injections. The remaining nymphs were maintained under the same conditions as described above. The visible phenotype changes were recorded every day until the nymphs started to molt to adults.

**Immunohistochemistry**—To analyze LmCDA2 protein localization, immunostaining was performed as described (33). In brief, paraffin sections (5 μm) of the third abdominal segments from nymphs 9 days after injection of dsGFP or dsLmCDA2 in 2-day-old 5th-instar nymphs were prepared. The LmCDA2 protein was detected in paraffin section with the *Drosophila* Verm rabbit antiserum (1:300) (16) as a primary antibody at 4 °C overnight followed by washing with PBS three times for 5 min each. Then tissues were incubated with Cy3-Affinipure donkey anti-rabbit (Jackson ImmunoResearch) as secondary antibody for 1 h at room temperature. After washing the tissues three times with PBS, specimens were incubated with Fluorescent Brightener 28 (FB28) (Sigma) (1 mg/ml) for 5 s to detect chitin (34). The preimmune rabbit serum was used as negative control. We verified that the *D. melanogaster* Verm antibody cross-reacts with LmCDA2 in Western blotting and mass spectrometry experiments as shown in supplemental Figs. 1–4. Images were captured on an LSM 880 confocal laser-scan.
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Author Contributions—J. Z. and B. M. conceived and coordinated the study and wrote the paper. R. Y. designed, performed, and analyzed the experiments shown in Figs. 1–4. W. L. designed, performed, and analyzed the experiments shown in Figs. 4 and 5. D. L., L. M., and R. L. designed, performed, and analyzed the experiments shown in Figs. 1–4. W. L. designed, performed, and analyzed the experiments shown in Figs. 1–4. R. Y. designed, performed, and analyzed the experiments shown in Figs. 1–4. All authors reviewed the results and approved the final version of the manuscript.

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