Molecular cloning and functional characterization of two insect chitin deacetylases from the American white moth, *Hyphantria cunea*

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Abstract
Chitin deacetylases (CDAs) convert chitin into chitosan and play crucial roles in the insect biological processes, including growth and development. In this study, two CDA-encoding genes, *HcCDA1* and *HcCDA4*, were identified from *Hyphantria cunea*. Conserved Domain Database for HcCDA1 identified a chitin-binding peritrophin-A domain (ChBD), a low-density lipoprotein receptor class A domain (LDLa), and a putative CDA-like catalytic domain (CDA), whereas HcCDA4 lacked the LDLa domain. Phylogenetic comparison with other insect CDAs revealed that HcCDA1 belonged to the insect group I CDA and HcCDA4 belonged to group III. qRT-PCR results showed that *HcCDA1* and *HcCDA4* were more highly expressed in newly moulted 5th-instar larvae, and *HcCDA1* was highly expressed in the integument, whereas *HcCDA4* was abundantly transcribed in the integument and hindgut. Western blot revealed that the expression of *HcCDA1* and *HcCDA4* in translational levels were consistent with the transcription. Double-stranded RNA injection of *HcCDA1* and *HcCDA4* into *H. cunea* larvae significantly reduced *HcCDA* transcript levels, resulted in larval–pupal moulting difficulty, and produced high larval mortality. These results demonstrate important contributions of HcCDA1 and HcCDA4 to insect moultling and development and provide potential new targets for designing effective double-stranded RNA-based pesticides to protect crops.

Keywords
Chitin deacetylases, *Hyphantria cunea*, insect moulting and development, RNA interference

1 | INTRODUCTION

Chitin, an N-acetyl-D-glucosamine polymer, is a major component of the insect cuticle and peritrophic matrix (Merzendorfer, 2003). Insect growth and development are intimately coupled with chitin biosynthesis, turnover and modification. Chitin remodelling maintains the dynamic balance of chitin, involves various insect enzymes and proteins, which are considered as important targets for pest control (Moussian, 2010; Muthukrishnan et al., 2016; Muthukrishnan et al., 2019; Qu et al., 2014; Terblanche, 2013; Zhu et al., 2016). Therefore, the study of key enzymes in chitin metabolism has become popular for finding new pest control targets (Chikate et al., 2016; Tetreau et al., 2015; Vogel et al., 2018).

Chitin deacetylases (CDAs) are metalloproteins that belong to an extracellular chitin-modifying enzyme family, and they deacetylate chitin to form chitosan (IasonTsigos et al., 2000).
are widely distributed and have been identified in different species, such as in Lepidoptera (Guo et al., 2005; Quan et al., 2013; Toprak et al., 2008), Diptera (Dixit et al., 2008), Coleoptera (Arakane et al., 2009), Hemiptera (Li et al., 2017; Xi et al., 2014) and Orthoptera (Yu, Liu, Li, et al., 2016; Yu, Liu, et al., 2019). Insect CDAs are divided into five groups on the basis of the functional domain (Dixit et al., 2008). Group I and II CDAs contain a chitin-binding peritrophin-A domain (ChBD), a low-density lipoprotein receptor class A domain (LDLa), and a putative CDA-like catalytic domain. The domain organizations of group I and group II are similar but with substantially different amino acid sequences. Group III and IV CDAs contain ChBD and CDA domains. Group V CDAs only contain a CDA domain (Arakane et al., 2009; Liu et al., 2019; Xi et al., 2014). In 2009, Arakane et al. characterized nine genes from Tribolium castaneum, and dsRNA-mediated knockdown of group I CDAs led to developmental disturbances and lethal phenotypes, whereas knockdown of all other CDAs did not result in any visible phenotypic changes (Arakane et al., 2009). Reports showed that group I CDAs (NiCDA1 and NiCDA2) and a group IV CDA (NiCDA4) from Nilaparvata lugens were highly expressed in the integument and periodically peaked during every moulting; injection with double-stranded RNA (dsRNA) for NiCDA1, NiCDA2, and NiCDA4 led to lethal phenotypes (Xi et al., 2014). In the migratory locust Locusta migratoria, RNA interference (RNAi) of LmCDA2 caused a less compacted and larger abdominal cuticle with fewer well-defined chitinous laminae (Yu, Liu, Li, et al., 2016). CDA2 knockdown in Leptinotarsa decemlineata affected chitin accumulation, decreased foliage consumption, prolonged larval development, and caused retarded larval growth (Wu et al., 2019). In Diaphorina citri, DcCDA3 was highly expressed in the integument and third-instar nymph stage, and it can be induced by 20E. In addition, recombinant DcCDA3 possessed antibacterial activity against gram-positive bacteria, which speculate that DcCDA3 might importantly related to the immune response of D. citri (Yu, Li, et al., 2019). RNA interference against Drosophila demonstrates that DmCDA1 and DmCDA2 have distinct functions during differentiation of the wing cuticle, and DmCDA2 is required for the laminar arrangement of chitin (Zhang, Ji, et al., 2019). In Bactrocera dorsalis, BdCDA1 and BdCDA2 have high expression during larval-pupa-adult moults, suggested their role involvement in metamorphosis of B. dorsalis. These findings suggest that group I CDAs function in chitin degradation in the integument and trachea of insects (Liu et al., 2018). RNA interference-mediated HvCDA1 and HvCDA2 led to abnormal or non-viable phenotypes in Heritiera littoralis and survival rates of the larvae have reduced, suggest that HvCDA1 and HvCDA2 play important roles in the larval-pupal and pupal-adult transitions (Wang et al., 2019). Double-stranded RNA (dsRNA)-mediated knockdown of Group I CDA genes in Tribolium castaneum causes that developmental disturbance and non-viable phenotypes (Noh et al., 2018). In Bombyx mori, the silencing of BmCDA1 and BmCDA2 did not lead to abnormal phenotypes or death but may have led to delays in silkworm pupation (Zhang, Yan, et al., 2019). These results indicate that CDAs may be suitable targets for RNAi-based insecticide development.

Previously, we reported that HcCDA2 plays an important role in insect moulting and development (Yan et al., 2018). In this study, to investigate the group I and III CDA function in insect cuticle organization, HcCDA1 and HcCDA4 were identified from the American white moth Hyphantria cunea and the transcription patterns at all larval development stages and in different tissues were assessed. For functional analyses, we examined the phenotypes of larvae and the organization of the cuticle after injection of dsRNA to downregulate the expression of HcCDA1 and HcCDA4. These experimental results could provide a foundation for developing new insecticide strategies to control H. cunea.

2 | MATERIALS AND METHODS

2.1 | Insect

Fall webworm larvae (H. cunea; Lepidoptera; Arctiidae) were purchased from the Chinese Academy of Forestry Science and reared on an artificial diet under a 16-hr light/8-hr dark photoperiod at 26 (±1)°C with 75 (±10)% relative humidity.

2.2 | Cloning of CDA genes from Hyphantria cunea

Total RNA was extracted from the integument of 5th-instar H. cunea with an RNAprep Pure Tissue Kit (Tiangen Co., Ltd) in accordance with the manufacturer’s instructions. The first-strand cDNA was synthesized with a First-stand cDNA Synthesis Kit (Promega) following the manufacturer’s protocol. A gene fragment of CDA1 from H. cunea was obtained by RT-PCR with primers cdaf1 F1/R1 (Table 1), which were designed on the basis of the conserved sequence of CDA1 from closely related species. Additional partial sequences were obtained by rapid amplification of cDNA ends (RACE) PCRs. The 5’ and 3’ partial sequences were amplified using antisense and sense gene-specific primers (Table 1). Specific primers of HcCDA1 (cda1 F2/R2, Table 1) were designed as per RACE products to obtain full-length cDNA. HcCDA1 was cloned into pMD18-T vector and sequenced. The cDNA sequence of HcCDA4 was searched from the midgut transcriptome of H. cunea. The full-length sequence of HcCDA4 was obtained with the primers cda4 F1/R1 (Table 1) by PCR, and then cloned into pMD18-T vector and sequenced. The resulting HcCDA1 and HcCDA4 sequences were submitted to GenBank.

2.3 | DNA and protein sequence analysis

The amino acid sequences of HcCDA1 and HcCDA4 were deduced by DNAMAN software package (Lynnon Biosoft). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used for signal peptide prediction. The ExPASy NetOGlyc 3.1 and NetNGlyc 1.0 servers (http://www.cbs.dtu.dk/services) were used to predict potential O- and N-glycosylation sites, respectively. Sequence homology analyses and
multiple sequence alignments were performed using NCBI/BLASTp and ClustalX, respectively. The phylogenetic tree, which included CDA protein sequences available in GenBank, was constructed using the neighbour-joining method with 1,000 bootstrap sampling replicates in Molecular Evolutionary Genetics Analysis (MEGA X).

2.4 Developmental and tissue-specific expression of HcCDA1 and HcCDA4

The 1st to 6th larvae and pupae and day 1 to day 5 of 5th-instar larvae were collected. Different tissues dissected from 5th-instar larvae were also collected, including the integument, foregut, midgut, hindgut, Malpighian tubules and fat body. Each sample included 3–5 individuals and was measured in biological triplicate. Total RNA was isolated using the RNAprep Pure Tissue Kit (Tiangen Co., Ltd) following the manufacturer’s instructions, and the first-stand cDNA was synthesized following the manufacturer’s instructions (Promega). The qRT-PCR was used to synthesise cDNA, and the first-stand cDNA was used for qRT-PCR analysis with 

2.5 RNAi experiments

The dsRNA synthesis and injection were carried out as described by Arakane et al., (2009). The gfp (GenBank Acc. No. KC896843) was used as a negative control for non-specific effects of dsRNA, and unique regions of HcCDA1 and HcCDA4 were chosen as templates for dsRNA synthesis. Primers for dsRNA synthesis are shown in Table 1. A T7 RiboMAX™ Express RNAi System (Promega) was used to synthesise dsRNA in accordance with the manufacturer’s instructions.

To administer the dsRNA, 1-day-old of 5th-instar larvae were injected with 3 μl of 10 μg dsRNA solution through the abdominal side between the fourth and fifth abdominal segments using a Hamilton Microliter. Each treatment was conducted with 30 insects. The larvae were maintained on a normal artificial diet, and their mortality was recorded at 3 days post-injection. The qRT-PCR was performed to analyse HcCDA1 and HcCDA4 gene-silencing efficiency. Total RNA was isolated 3 days after dsRNA injection. Three biological replicates were prepared to perform qRT-PCR samples and the qRT-PCR experiment consisted of three technical replicates. Western blot was used to assess HcCDA1 and HcCDA4 knockdown. Immunohistochemistry was used to analyse HcCDA1 and HcCDA4 expression levels after RNAi. Serial paraffin sections were cut as described by Yu et al. (Yu, Liu, Li, et al., 2016). Paraffin sections (6 μm) of transverse sections from larvae 3 days after injection of dsRNA in 5th-instar larvae were prepared. The sections were blocked with BSA, combined with HcCDA-specific antibody, and then incubated with FITC conjugated specific antibody (Boster). Finally, the tissues were incubated with FB28 (Sigma). Fluorescence was viewed with an OLYMPUS FLUOVIEW FY1000 confocal laser-scanning microscope equipped with a UV (405 nm) and a VIS (488 nm) laser to excite FB28 and FITC anti-rabbit antibodies (Boster), respectively.

After injection with dsRNA of gfp/-HcCDA1/-HcCDA4 for 3 days, the whole body of H. cunea was collected and oven-dried at 90°C to constant weight, and chitin content was determined as previously described (Zhang & Zhu, 2006). Glucosamine was used for standard

| Table 1 Primers used in this study |
|-----------------------------------|
| Primer | Forward (5′-3′) | Reverse (5′-3′) |
| cda1 F1/R1 | CAACGATGACAGCGCTTCT | CACGTCTGCAGGTGATGGT |
| 5′ GSP | CTGAAGTACATTGTGTAGGGCCATAGGGGTG | – |
| 3′ GSP | CAGTACTATTACAGCTCCCTTATCAGATCCACC | – |
| cda1 F2/R2 | ATGGCGGCTCTACGCCGGTTGCTGCT | ATAAAGGCGCTACCTGTAGGATACA |
| cda4 F/R | ATGTCGATACACACAGTGTTACTTCCTTCTGAGGTTTTCTGGTAC | GACACAGTGGCTCATCACAG |
| qcd1a F/R | CCAGCTGGTTTGTACTTTTG | ATACCGATCTTTCACCCAC |
| qcd4a F/R | CAACCGGCTTCTTCATACAGAG | ACTCCTCCTCAGGTTTCTGAC |
| qactin F/R | CTACCTCAGCGCTTCTTC | AGCTTCTCCTGAGTCTAC |
| dsCDA1 F/R | TAATACGACTCATATAAGGTGATCCGATTTCTG | TAATAGCAGCTCATAAGGTGATCCGATTTCTG |
| dsCDA4 F/R | TAATACGACTCATATAAGGTGATCCGATTTCTG | TAATACGACTCATATAAGGTGATCCGATTTCTG |
| dsGFP F/R | TAATACGACTCATATAAGGTGATCCGATTTCTG | TAATACGACTCATATAAGGTGATCCGATTTCTG |

The expression levels of HcCDA1 and HcCDA4 were assessed by Western blot with HcCDA1- and HcCDA4-specific antibodies, which were prepared by the Institute of Biology, Hebei Academy of Sciences (Shijiazhuan, HeBei) using activated and purified HcCDA1 and HcCDA4 proteins as antigens. The HcACTIN-specific antibodies were prepared as described above and used to normalize the protein samples (Yan et al., 2018). The experiment comprised three technical replicates.

The 2 and the qRT-PCR experiment comprised three technical replicates. Three biological replicates were performed to prepare RNA samples and the qRT-PCR was carried out on a Bio-Rad CFX machine (Bio-Rad). The qRT-PCR was used to analyse the transcript expression levels, with hcactin as a reference gene. Primers synthesized following the manufacturer’s instructions (Promega) and was used for qRT-PCR analysis. The reaction volume that contained 1 μl SYBR Ex Taq (Japan, TaKaRa), and was carried out on a Bio-Rad CFX machine (Bio-Rad). Three biological replicates were performed to prepare RNA samples and the qRT-PCR experiment comprised three technical replicates. The 2^ΔΔCt method was used to evaluate the quantitative variation (Livak & Schmittgen, 2001).

The expression levels of HcCDA1 and HcCDA4 were assessed by Western blot with HcCDA1- and HcCDA4-specific antibodies, which were prepared by the Institute of Biology, Hebei Academy of Sciences (Shijiazhuan, HeBei) using activated and purified HcCDA1 and HcCDA4 proteins as antigens. The HcACTIN-specific antibodies were prepared as described above and used to normalize the protein samples (Yan et al., 2018). The experiment comprised three technical replicates.

The expression levels of HcCDA1 and HcCDA4 were assessed by Western blot with HcCDA1- and HcCDA4-specific antibodies, which were prepared by the Institute of Biology, Hebei Academy of Sciences (Shijiazhuan, HeBei) using activated and purified HcCDA1 and HcCDA4 proteins as antigens. The HcACTIN-specific antibodies were prepared as described above and used to normalize the protein samples (Yan et al., 2018). The experiment comprised three technical replicates.
curve preparation. Chitin content definition: chitin (mg)/tissue weight (g) = glucosamine concentration (μg/ml)/tissue weight (g).

3 | RESULTS

3.1 | Analysis of HcCDA1 and HcCDA4 cDNA and deduced amino acid sequences

Full-length HcCDA1 and HcCDA4 sequences were obtained by reverse transcription-quantitative PCR (RT-qPCR) and submitted to GenBank under accession numbers KF975504 and KX822766, respectively. The open reading frames of HcCDA1 and HcCDA4 were 1632 bp and 1494 bp, which encoded 543 and 497 amino acids, respectively. The predicted molecular weights of HcCDA1 and HcCDA4 were 62 kDa and 56 kDa, respectively. The secreted HcCDA1 contained four putative N-glycosylation sites at positions 35(NATA), 248(NYSA), 278(NATV) and 300(NITD), while HcCDA4 contained two putative N-glycosylation sites at positions 248(NISR) and 458(NFTD), neither of them has O-glycosylation site. A search of the Conserved Domain Database (NCBI) for HcCDA1 identified a signal peptide, a chitin-binding peritrophin-A domain (ChBD), a low-density lipoprotein receptor class A domain (LDLa), and a putative CDA-like catalytic domain (CDA), whereas HcCDA4 lacked the LDLa domain and only contained a signal peptide, a ChBD, and a CDA domain (Figure 1).

Phylogenetic analysis was performed with the CDA-like protein sequences from other insect species. The neighbour-joining method was used to construct a phylogenetic tree, and the results showed that there were five CDA groups (groups I to V): HcCDA1 belonged to group I CDA, whose members contain all three domains (CDA, ChBD, and LDLa), and a putative CDA-like catalytic domain (CDA), whereas HcCDA4 lacked the LDLa domain and only contained a signal peptide, a ChBD, and a CDA domain (Figure 1).

FIGURE 1 | Alignment of deduced amino acid sequences of HcCDA1 and HcCDA4. The predicted signal peptide is underlined. The blue and green boxes indicate a chitin-binding peritrophin-A domain and low-density lipoprotein receptor class A domain, respectively. The CDA catalytic domain is double underlined [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 | Phylogenetic analysis of known insect CDAs. The accession number and species abbreviations of CDAs are as follows: Hc: Hyphantria cunea, Bm: Bombyx mori, Ld: Leptinotarsa decemlineata, Dm: Drosophila melanogaster, Tc: Tribolium castaneum, Oc: Oxya chinensis, Cf: Choristoneura fumiferana, Ob: Operophtera brumata, Ha: Helicoverpa armigera, Mb: Mamestra brassicae, Dp: Danaus plexippus, Mc: Mamestra configurata Mamestracocon figurata, Ni: Nilaparvata lugens, Cm: Cnaphalocrocis medinalis. HcCDA1 and HcCDA4 are shaded [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Developmental and tissue-specific mRNA expression of HcCDA1 and HcCDA4

Developmental and tissue-specific expression of HcCDA1 and HcCDA4 was determined by qRT-PCR (Figure 3). HcCDA1 and HcCDA4 were more highly expressed in 5th-instar larvae, and the stage-dependent expression pattern in the 5th-instar larvae on different days showed that the highest expression occurred in newly moulted larvae. Tissue-specific expression results showed that HcCDA1 was highly expressed in the integument, whereas HcCDA4 was abundantly transcribed in the integument and hindgut.

3.3 | Expression patterns of HcCDA1 and HcCDA4

The expression patterns of HcCDA1 and HcCDA4 protein were studied by Western blot with specific antibodies (Figure 4). HcCDA1 and HcCDA4 were both expressed in different developmental stages of H. cunea, and abundantly expressed in the 1-day and 2-day-old 5th-instar larvae. Tissue-specific expression results showed that HcCDA1 and HcCDA4 were detected in the head, integument and hindgut tissues. HcCDA1 and HcCDA4 expression levels were consistent with the transcriptional levels.

3.4 | Effects of HcCDA-directed RNAi on moulting and metamorphosis

To investigate the functions of HcCDA1 and HcCDA4 in moulting, double-stranded RNAs (dsRNAs) for HcCDA1 and HcCDA4 were injected into the 1-day-old of 5th-instar larvae. dsRNA-green fluorescent
protein (gfp) was used as a control. Results showed that the larvae injected with ds gfp showed normal behaviour, and they could successfully pupate, whereas the dsHcCDA1- and dsHcCDA4-injected larvae underwent moulting difficulty, showed little movement, and most failed to shed the old cuticle; the survivors displayed slowed development and abnormal pupation (Figure 5a). The mortalities of larvae injected with dsHcCDA1 and dsHcCDA4 were 93% and 87%, respectively. The HcCDA genes were substantially downregulated and compared with the ds gfp-injected controls, the expression levels of HcCDA1 and HcCDA4 in larvae were reduced by approximately 90.3% and 84.9% of silencing efficiency (Figure 5b). Western blot results revealed that HcCDA1 and HcCDA4 were knocked down after gene expression was inhibited (Figure 5c).

A positive signal was found in the cuticles of the ds gfp-injected H. cunea larvae. This signal was visible at the apical site of the procuticle, but only a weak signal was detected in the dsHcCDA1-and dsHcCDA4-injected larvae (Figure 5d). The chitin amounts were significantly decreased in dsHcCDA1-and dsHcCDA4-injected insects compared with ds gfp-injected larvae (Figure 5e). These results indicate that HcCDA1 and HcCDA4 play vital roles in H. cunea development and moulting.

4 | DISCUSSION

CDAs are important enzymes that regulate moulting, organ formation and antipathogen immunity during insect development (Dixit...
Insect CDAs are divided into gut-specific CDAs (group V CDAs) and non-intestinal-specific CDAs (group I, II, III and IV CDAs), and they are expressed in specific tissues and developmental stages of different insects. The group I CDAs were shown to modify cuticular and tracheal chitin in Drosophila melanogaster and T. castaneum, and are periodically expressed during insect moulting in N. lugens and L. migratoria (Arakane et al., 2009; Luschnig et al., 2006; Yu, Liu, Li, et al., 2016). The group III CDAs are mainly expressed in the integument, foregut, and hindgut tissues and are highly expressed in the 1-day and 2-day-old of 5th-instar L. migratoria nymphs. In this work, we obtained two CDA-encoding genes, HcCDA1 and HcCDA4, functional domain analysis and phylogenetic analysis revealed that HcCDA1 contains ChBD, LDLa and CDA domains, and belonged to group I, whereas HcCDA4 only has ChBD and CDA domains, and belonged to group III. HcCDA1 and HcCDA4 were more highly expressed in the 1-day and 2-day-old of 5th-instar larvae, and tissue expression results showed that HcCDA1 was highly expressed in the integument, whereas HcCDA4 was abundantly transcribed in the integument and hindgut. We also detected the HcCDA1 and HcCDA4 protein expression levels, which were consistent with the transcriptional levels.

To date, the biological functions of group I CDAs have been extensively detected, whereas the specific roles of the other group CDAs need further research. Using RNAi to silence CDA1 and CDA2 in T. castaneum resulted in molting arrest and subsequent mortality (Arakane et al., 2009; Terenius et al., 2011). Similarly, injection of dsRNA that targeted LmCDA2 in L. migratoria nymphs arrested 5th-instar eclosure, the insects were unable to moult and eventually died (Yu, Liu, Li, et al., 2016). Moreover, HcCDA2a from H. cunea plays a crucial role in insect development (Yan et al., 2018). These studies revealed that group I CDAs are potential targets for pest control, as these proteins can influence the development and moulting of insects (Yu, Liu, Wang, et al., 2016; Yu, Liu, Li, et al., 2016). However, there is no conclusions regarding group III CDAs’ function could be drawn, group III TcCDA4 in T. castaneum is expressed in early imaginal appendages, but no adverse consequences were detected after RNAi (Arakane et al., 2009; Muthukrishnan et al., 2016). RNAi of NICDA3 in group III did not result in any abnormal moulting or other observable morphological or interstructural abnormalities, a possible explanation is that NICDA3 might contribute to the defence of insects against pathogens (Dixit et al., 2008). In this study, injection of dsRNA-HcCDA1 led to the shedding failure of old cuticles and high mortality, which provides strong evidence for its critical role in molting. A lethal phenotype after dsRNA-HcCDA4 injection was observed and chitin content were reduced, these were different from what was observed in N. lugens and T. castaneum, showed no observable morphological phenotype (Arakane et al., 2009; Xi et al., 2014). In the present study, injection of dsHcCDA4 led to failure to shed old cuticles and high mortality, which indicates a critical role in moulting and group III HcCDA are essential for insect survival.

In summary, HcCDA1 and HcCDA4 were identified from H. cunea, and grouped into CDA groups I and III, respectively. We demonstrated that HcCDA1 and HcCDA4, which encode CDA-like proteins, are present and expressed in H. cunea; however, their expression patterns differed among developmental stages and tissues. The biological functions of group III CDAs in H. cunea were first assigned, and RNAi results revealed that group I and III CDAs are involved in functions associated with insect molting by modifying chitin in the embryonic stages. Understanding the function of HcCDAs will provide a foundation for developing new insect control strategies for H. cunea and define potential targets for RNAi-based pest management.

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FIGURE 5 Effects of dsHcCDA1 and dsHcCDA4 on Hyphantria cunea larvae. (a) Phenotypes of insects injected with dsRNA-gfp/-HcCDA1/-HcCDA4; dsgfp was used as a negative control. The larvae injected with dsRNA-gfp underwent normal moulting and successful pupation, whereas the dsHcCDA1- and dsHcCDA4-injected insects displayed abnormal moulting and pupation phenotypes. (b) Silencing efficiency after injection with dsHcCDA1 and dsHcCDA4 as determined by qRT-PCR analysis. Transcript levels were normalized with hcactin. The data were given as means ± SE of three different injection experiments. Asterisks on the columns indicate significant differences between the control and dsRNA injection treatment groups (p < .05). (c) Western blot expression analysis of HcCDA1 and HcCDA4 after dsRNA injection. (d) Immunofluorescence detection of HcCDA1 and HcCDA4 after RNAi (green, FITC; blue, FB28). Scale bar, 5 µm. (e) Chitin content detection of H. cunea larvae injected with dsRNA-gfp/-HcCDA1/-HcCDA4. Asterisks on the columns indicate significant differences between the control and dsRNA injection treatment groups (p < .05) [Colour figure can be viewed at wileyonlinelibrary.com]
CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Dan Zhao, Han Wu and Wei Guo conceived the research. Dan Zhao, Han Wu, Xiaoping Yan and Yazi Li conducted experiments. Dan Zhao and Wei Guo contributed material. Xiujun Lu, Xiaochang Guo and Zhaorui Liu analysed data and conducted statistical analyses. Dan Zhao wrote the manuscript. Wei Guo secured funding. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT
The data supporting this manuscript are available at: https://zenodo.org/record/4444849.

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