SCIENTIFIC REPORTS

OPEN

Received: 29 March 2017
Accepted: 13 November 2017
Published online: 22 November 2017

Isolation and functional identification of three cuticle protein genes during metamorphosis of the beet armyworm, *Spodoptera exigua*

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The beet armyworm, *Spodoptera exigua* (Hubner), is one of the major crop pests and is a target for current pest control approaches using insecticides. In this study three cuticular protein genes *CPG316*, *CPG860* and *CPG4855* have been cloned from 0 h pupal integument of *S. exigua* through race PCR Strategy. The deduced amino acid sequences were found to contain the RR-2 consensus region of other insect cuticular proteins and construct phylogenetic trees for each protein. Using quantitative RT-PCR, the developmental expression of the three genes through several larval and the early pupal stages was studied. All three genes contribute to the endocuticle although *CPG316* may have a different role from the other two genes. All three newly isolated genes were analyzed and their functions were determined by using direct injection of the dsRNA into early 5th instar larvae. All genes are expressed in the larvae and early pupae but in different patterns. Furthermore, phenotypic results show that these genes have differing effects on the development of cuticle, its flexibility and a big role in metamorphosis in both larval and pupal stages.

The insect cuticle is a multi-layered structure with three functional regions, the epicuticle, the exocuticle, and endocuticle, which differ in protein composition, structure, and functions[1]. The structure of the cuticle is determined by the cuticular proteins. Many types of cuticle show prominent differences in mechanical properties and these differences are related to the properties of the individual proteins[2]. The properties of insect cuticle differ in different stages and species due to the mixture of cuticular proteins. The cuticle performs important roles in many physiological conditions and defends the insect from water loss, penetration of insecticides, and protect from physical injury and pathogens[3–6]. Many conserved motifs in cuticular proteins[5] have been identified including Rebers and Riddiford (R&R) Consensus[7]. Proteins with R&R Consensus can be split into three groups, RR-1, RR-2, and RR-3, with some correlation to the type or region of the cuticle. The cuticle proteins with 44-amino acid motifs belong to (CPF)[8], and Tweedle[9] consensus. Among them, the cuticular protein sequences containing R&R Consensus (CPR) were broadly studied in *Anopheles gambiae, Drosophila melanogaster, Bombyx mori* and *Apis mellifera* by the annotation of genomic data[10–13]. Many cuticular protein genes have been isolated, and their ecdysone-responsive characteristics have been studied[14–20].

The beet armyworm, *Spodoptera exigua* (Hübner), is a destructive pest of vegetables and crops[14]. This pest has a long history of being treated with insecticides, which have resulted in the development of resistance to a diverse array of chemical classes[21–27]. The life cycle of this pest consists of four different stages: eggs, five larval instars, pupae, and adults[28]. The pupal melanic strain of *Spodoptera exigua* (SEM) with increased fitness was found in a

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laboratory population[29]. Transcriptome analysis between the SEM strain and wild-type strain (SEW) revealed several cuticular protein genes overexpressed at 0 h pupa stage (unpublished data).

The substitute biotechnological techniques depend mainly on *Bacillus thuringiensis* (BT) expression of insecticidal protein Cry toxins. Nevertheless, there has been a development of resistance to these toxins in some of the species such as (*Heliothis virescens*, Lepidoptera: *Noctuidae*) and (*Ostrinia nubilalis*, Lepidoptera *Pyralidae*)[30,31]. RNAi was first used on the function of insect gene as a highly effective tool for the research purpose of the fruits fly *D. melanogaster* as a model pest[32]. RNAi technology and the elucidation have set for the development and their growth in the RNAi systems and application of this in *D. melanogaster* lately[33] have reviewed the mechanisms of RNAi uptake in insects body and its possible potential for insect management[34] RNAi was described by Fire, *et al.*[35]. The post-transcriptional gene silencing ability within target insect with dsRNA double-strand RNAs method was introduced by Geley & Müller[36].

To advance understanding of the molecular genetics basis of the overexpressed cuticular protein genes in the beet armyworm, we have isolated and characterized three cuticular protein genes from 0 h pupae, as well as the temporal expression profiles of these three genes was observed. The results showed that these three cuticular protein genes had the RR-2 motif consensus sequence based on the conserved motif and phylogenetic tree analysis. The *CPG316* gene was expressed abundantly at the 2 h pupa stage while the *CPG860* and *CPG4855* showed decreasing expression during development from the 3rd instar to the 24 h pupa. The differential expression patterns of these genes may reveal their property to be utilized in a novel control strategy. We used RNAi to observe the function of these genes determined using direct injection of three different concentrations (100 ng/μl, 200 ng/μl and 500 ng/μl) of the dsRNA into early 5th instar larvae. The results revealed that these genes have differing effects on the cuticle in both larval and pupal stages and are required for cuticle development and normal metamorphosis.

Materials and Methods

Insects. *S. exigua* larvae from Jingzhou Hubei Province, China, were maintained with artificial diet in the laboratory at 27 °C and 65% relative humidity and a photoperiod of 14L:10D[37].

Gene cloning. RNA was extracted from 0 h pupae integument. The cDNA was synthesized as suggested in the SMART RACE kit (Takara, Biotech, Japan). Gene-specific primers (GSP1) and nested gene-specific primers (NGSP2) were designed for 5′- and 3′-RACE using the software Primer Premier 5.0. The primer sequences are listed in (Table S1). The first round PCRs were performed with the GSP primer and Universal Primer Mix (UPM). The 99X diluted first PCR products were used as the templates in the nested PCRs with NGSP. The RACE products were separated on an agarose gel and purified using the AXYGEN Gel and PCR Clean-Up System (Promega, USA). Purified cDNA was ligated into PMD18-T Vector (Takara, Biotech, Japan) and sequenced completely from both directions with the equivalent specific primers (Table S1) designed based on their partial cDNA sequences. The open reading frames (ORF) of *CPG316, CPG860*, and *CPG4855* were amplified from *S. exigua* with the corresponding PCR primers (Table S1), respectively. The ORF PCR conditions for *CPG316, CPG860*, and *CPG 4855* genes were 3 min at 95 °C, followed by 40 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 59 °C, 60 °C and 58 °C, respectively, and 1 min extension at 72 °C and a final 10 min extension at 72 °C. The flanking PCR conditions follow the same procedure as ORF PCR. The full ORFs were cloned into pMD18-T Vector (Takara, Biotech, Japan) and at least three clones were sequenced for each fragment[38].

Sequence alignment and phylogenetic analysis. The amino acid sequences were blasted in NCBI database (https://www.ncbi.nlm.nih.gov/) using the blast-p option. The similar amino acid sequences were retrieved to use for construction of the phylogenetic tree with MEGA 7.0 and the same sequence aligned using ClustalX 2.1 with default settings and visualized in GeneDoc to show the conserved amino acids[39]. The percent similarity and identity were determined using (http://danio.mgh.harvard.edu/blast/wblast2.cgi?0) among the three cuticle protein genes *CPG316, CPG860*, and *CPG4855* and those of other insect species.

Real-time PCR. Total RNA was isolated from the dissected integuments at six-time points during the intermolt period (endocuticle being synthesized) (3rd instar, 4th instar, 5th instar, 0 h pupa, 2 h pupa, 24 h pupa) and reverse transcribed into cDNA as described above. The cDNAs were used as the templates for quantitative RT-PCR (qRT-PCR) analysis of *CPG316, CPG860*, and *CPG4855* expression using SYBR® Premix Ex TaqTM II kit (Takara, Biotech, Japan). Real-time PCR of *CPG316*, *CPG860*, *CPG4855*, *β-actin*, and *GAPDH* (*β-actin* and *GAPDH* were internal reference genes) were performed individually in a 20 μL reaction containing 2× SYBR® Premix Ex TaqTM II 10 μL, 10 μM forward primer and reverse primer (0.8 μL each), 1 μL template cDNA, and 7.4 μL nuclease-free water. Specific primers (Table S1) were designed for real-time PCR of *CPG316, CPG860, CPG4855, β-actin* and *GAPDH* to generate the corresponding amplicons of 102 bp, 144 bp, 116 bp, 107 bp, and 174 bp, respectively. All real-time PCR reactions were performed in VIOX/SCIENTIFIC 96-well PCR plates. qTOWER 2.0 & 2.2 by Analytik Jena real-time PCR detection system was used as the fluorescence detector with the following common PCR conditions for the three genes: an initial denaturing cycle of 95 °C for 30 sec, followed by 40 cycles of denaturation at 95 °C for 5 sec, annealing at 60 °C for 10 sec and extension at 72 °C for 30 sec, and data collection and real-time analysis enabled at 72 °C. Melting curve analysis from 65 °C to 95 °C was run for each target and reference gene to ensure the absence of junk products. For the time points of 3rd instar, 4th instar, 5th instar, 0 h pupa, 2 h pupa, 24 h pupa, there were three biological replicates of three integuments each for each gene, and each biological replicate was qRT-PCR-analyzed for three times. The expression levels of these three genes (*CPG316, CPG860, CPG4855*) at each time point were calculated and normalized to the geometric mean of the expression of the two reference genes (*β-actin* and *GAPDH*) with the $2-\Delta Ct$ method, where $\Delta Ct = Ct$ target gene$-$ Ct reference gene, and Ct refers to the cycle threshold of the gene[30,32].

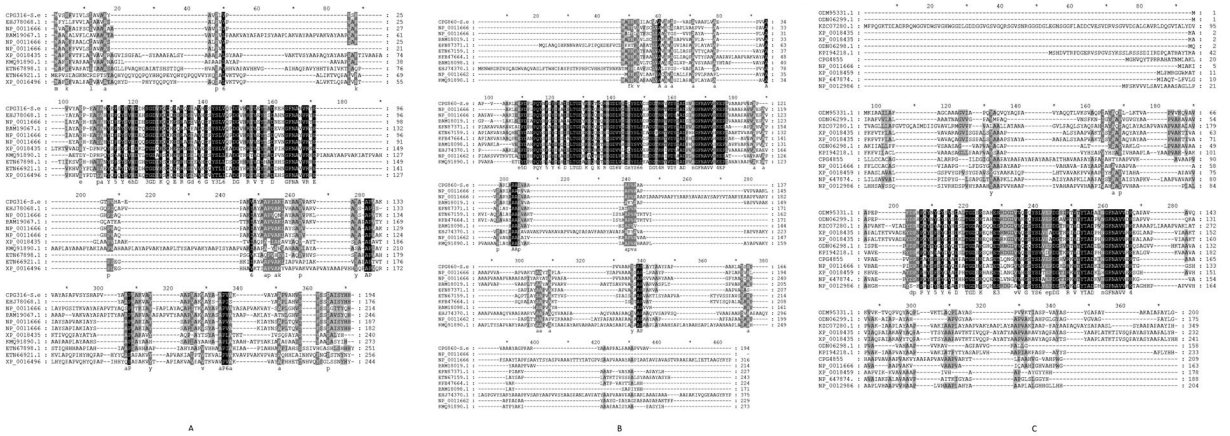

Figure 1. (**A**) Multiple alignments of the conserved region of the deduced amino acid sequence of *CPG316* with those of 331 other insect species. Black Shaded 100% conserved sequences are shown in the figure are active binding sites. (**B**) Multiple alignments of a conserved region of the deduced amino acid sequence of *CPG860* with those of 331 other insect species. Black Shaded 100% conserved sequences are shown in the figure are active binding sites. (**C**) Multiple alignments of the conserved region of the deduced amino acid sequence of *CPG4855* with those of 331 other insect species. Black Shaded 100% conserved sequences are shown in the figure are active binding sites.

dsRNA synthesis. According to the cloned ORF sequences of *CPG316, CPG860*, and *CPG4855* genes, we designed two pairs of gene-specific primers sense and antisense. The plasmid was extracted as a template, and then used the universal T7 promoter primer the sequence of amplified template for the synthesis of dsRNA with T7 RiboMAX™ Express RNAi System kit (Promega, USA). The same procedure was followed for dsRED as control, Sense primer pGEMTeasy with a T7 promoter sequence and antisense primer pGEMTeasy, follow by next pair of primers sense primer pGEMTeasy and antisense primer pGEMTeasy with the T7 promoter sequence. The dsRed reference template provided by the University of Arizona laboratory of Dr. Xianchun Li. The dsRNA were then purified with MEGA clear TM Kit (Ambion, USA).

dsRNA injection. The 5th instar beet armyworm larvae were starved for 24 h, then injected in the 2nd to last dorsal abdominal segment. Two µl dsRNA of three different concentrations (100 ng/µl and 200 ng/µl, 500 ng/µl) for each cuticle gene, 500 ng/µl dsRed (control), were injected. Thirty insects were treated with each concentration; each treatment set was repeated 3 times. Phenotypes were observed after 96 h and the mortality of the treated insects was recorded daily.

After 96 h of treatment, three abnormal insects were selected for each gene and controls. The phenotypes of these samples were imaged with a digital camera after seven days with a high-resolution microscope (OLYMPUS SZX16).

Statistical analysis. Data are presented as mean ± SD. One-way ANOVA with Duncan test was performed to test the significance of differences. Values sharing the different letters are significantly different at $P < 0.05$ (Duncan test). All the statistical tests were performed by SPSS (16.0).

Results

Sequence characterization. The full-length cDNA sequence of *CPG316* was deposited in the gene bank, which was assigned with a KY554477 accession number. The *S. exigua CPG316* gene (722 bp) contained a 43 bp 5′UTR (untranslated region), an open reading frame (ORF) of 585 bp encoding 194 amino acids, and a 94 bp 3′UTR (Fig S1A). The corresponding amino acid sequence was searched against BLAST (https://www.ncbi.nlm.nih.gov/) use blast-p. Multiple alignments of *CPG316* conducted by Gendoc. The results revealed that it has the following identities and similarities with other cuticular proteins as follows: 69% identity and 84% similarity with *Bombyx mori* (NP_001166684.1), 68% identity and 83% similarity with *B. mori* (NP_001166633.1), 69% identity and 82% similarity with *Danaus plexippus* (EHJ78068.1), 64% identity and 75% similarity with *B. mori* (NP_001166683.1), 63% identity and 73% similarity with *Papilio polytes* (BAM19067.1), 45% identity and 55% similarity with *Anopheles darlingi* (ETN66921.1), 48% identity and 63% similarity with *A. darlingi* (ETN67898.1), 45% identity and 55% similarity with *Aedes aegypti* (XP_001649697.1), 44% identity and 54% similarity with *Culex quinquefasciatus* (XP_0018435071), and 41% identity and 52% similarity with *Lasius niger* (KMQ91890.1) (Fig. 1A).

The full-length cDNA sequence of *S. exigua CPG860* was translated and analyzed as described above for *CPG316*. The *CPG860* having accession code (KY554478), cDNA sequence (710 bp in total) contains a 49 bp 5′UTR, an open reading frame of 585 bp encoding 194 amino acids, and a 76 bp 3′UTR (Fig. S1B). From alignment of a region of conserved amino acids, *CPG860 S. exigua* have 68% identity and 73% similarity with *B. mori* (NP_001166689.1), 68% identity and 71% similarity with *Bombyx mori* (NP_001166690.1), 66% identity and 73% similarity with *P. xuthus* (BAM18019.1), 60% identity and 65% similarity with *D. plexippus* (EHJ74370.1), 56%

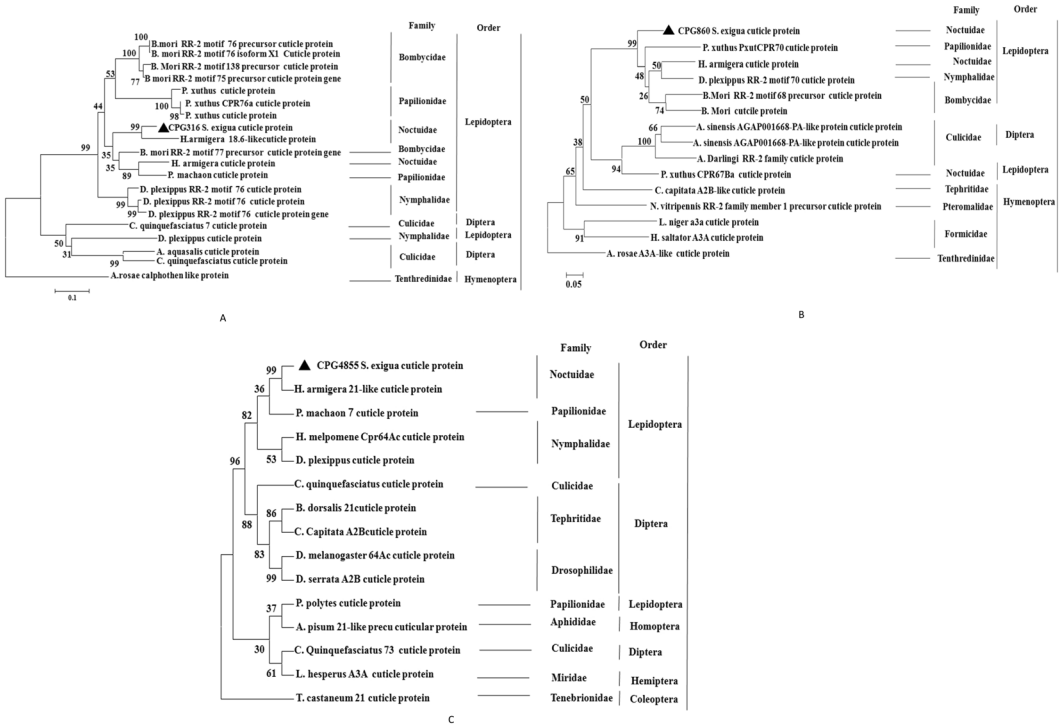

Figure 2. (**A**) Phylogenetic tree of insect *CPG316* based on its amino acid sequence. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The numbers at each tree node are the bootstrap values. (**B**) Phylogenetic tree of insect *CPG860* based on its amino acid sequence. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The numbers at each tree node are the bootstrap values. (**C**) Phylogenetic tree of insect *CPG4855* based on its amino acid sequence. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The numbers at each tree node are the bootstrap values.

identity and 65% similarity with *Harpegnathos saltator* (EFN87371.1), 55% identity and 64% similarity with *P. xuthus* (BAM18098.1), 53% identity and 61% similarity with *Nasonia vitripennis* (NP_001166273.1), 52% identity and 62% similarity with *L. niger* (KMQ91890.1), 50% identity and 50% similarity with *Anopheles sinensis* (KFB47664.1), 49% identity and 60% similarity with *A. darling* (ETN67159.1) (Fig. 1B).

The same procedure was followed for *S. exigua CPG4855*. The *CPG4855*, cDNA (accession code KY554479) and (868 bp in total) contains a 43 bp 5′UTR, consist of 630 bp ORF encoding 209 amino acids, and a 192 bp 3′UTR (Fig. S1C). Protein alignment analysis of *CPG4855* as done for the other two genes (Fig. 1C) shows that this gene has 68% identity and 74% similarity with *Papilio machaon* (KPJ08284.1), 67% identity and 72% similarity with *B. mori* (NP_001166686.1), 65% identity and 76% similarity with *Heliconius melpomene* (CBH09302.1), 65% identity and 74% similarity with *D. plexippus* (EHJ70419.1), 59% identity and 65% similarity with *Drosophila melanogaster* (NP_647874.1), 56% identity and 63% similarity with *C. quinquefasciatus* (XP_001845930.1), 52% identity and 60% similarity with *P. polytes* (NP_001298679.1), and 50% identity and 55% similarity with *Orchesella cincta* (NP_647874.1).

Phylogenetic analysis. The sequence of the conserved domain of *CPG316* is found in the same clade with Helicoverpa armigera, *B. mori* and *P. machaon* cuticle protein (Fig. 2A). The conserved region encoded by *CPG860* was found in the same clade with *P. xuthus, H. armigera, D. plexipus*, and *B. mori* cuticle proteins (Fig. 2B). The *CPG4855* was placed in the same clade with *H. armigera*, but little closed to *P. machaon* (Fig. 2C).

Developmental expression. *CPG316, CPG860*, and *CPG4855* transcripts were quantified in the qTOWER 2.0 & 2.2 by Analytik Jena real-time PCR detection system (Applied Biosystems). *Actin* and *GAPDH* genes were used as internal reference genes. All three genes were expressed at six-time points: 3rd, 4th, and 5th instar larvae (L3, L4, and L5) and 0 h, 2 h, 24 h pupae (P 0 h, P 2 h, and P 24 h) (Fig. 3A,B,C). The results revealed significant differences in the normalized *CPG316* expression among the six developmental time points (Fig. 3A). *CPG316* is expressed in the 4th instar larvae through the 0 h pupae at nearly constant levels, then increases in the 2 h pupae, only to fall again to a low level in the 24 h pupa that is similar to that in the 3rd instar larvae. *CPG860* is expressed in all stages at 10-fold higher normalized levels than is *CPG316*. The highest level is observed in the 3rd and 4th instar larvae, intermediate level in the 5th instar larvae to 2 h pupae and very low level in 24 h pupae (Fig. 3B). The level of normalized expression of *CPG4855* (Fig. 3C) was similar to that of *CPG860*. It is also highly expressed in

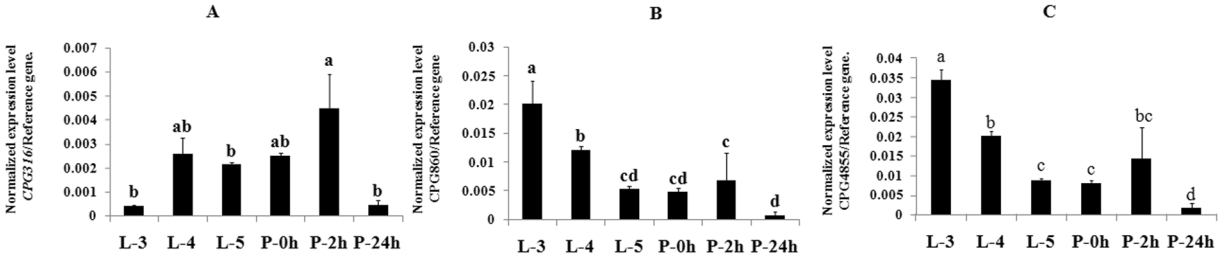

Figure 3. (**A**) Developmental expression of *CPG316* of *S.exigua*. The data and error bars represent the means and standard deviations of three biological replicates of three technical repeats each. Values sharing the same letter are not significantly different at $P < 0.05$ (Duncan test). (**B**) Developmental expression of *CPG860* of *S.exigua*. The data and error bars represent the means and standard deviations of three biological replicates of three technical repeats each. Values sharing the same letter are not significantly different at $P < 0.05$ (Duncan test). (**C**) Developmental expression of *CPG4855* of *S. exigua*. The data and error bars represent the means and standard deviations of three biological replicates of three technical repeats each. Values sharing the same letter are not significantly different at $P < 0.05$ (Duncan test).

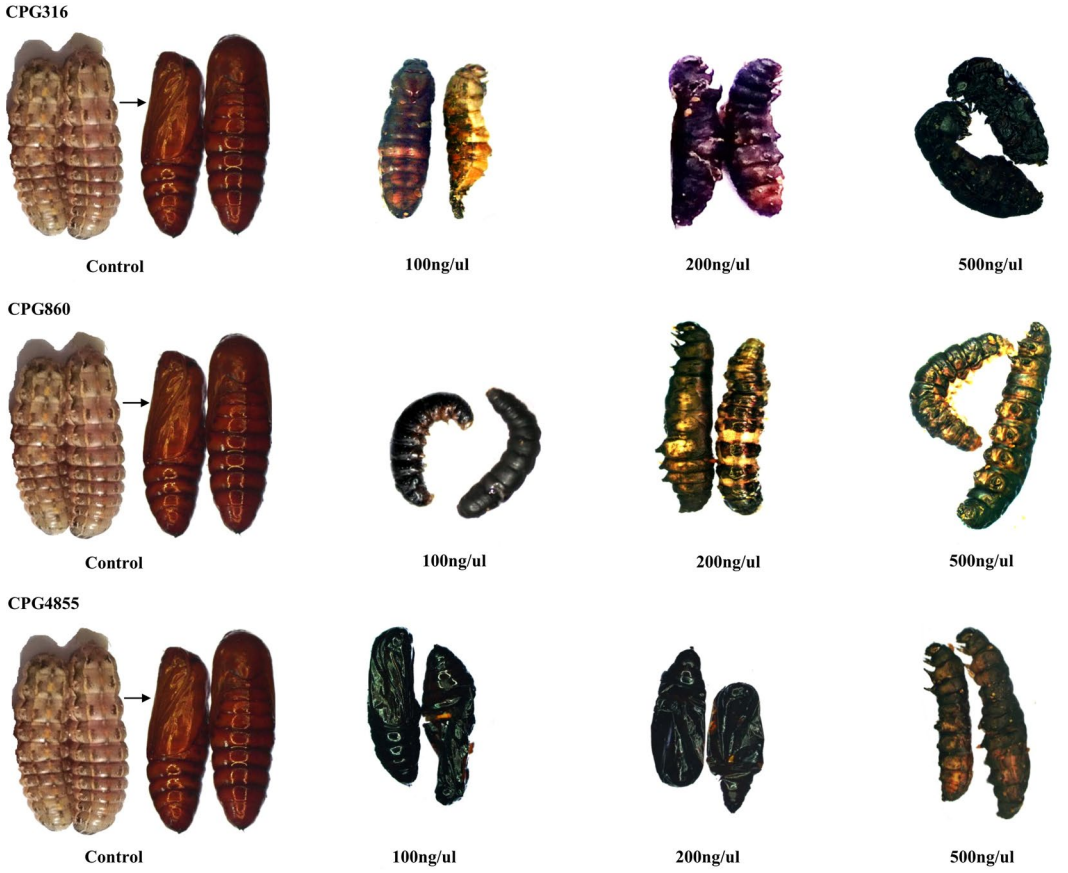

Figure 4. All genes phenotypes associated with three different concentrations of dsRNA as well (dsRed) as a control. (All three genes were separated with there names *CPG316*, *CPG860* and *CPG4855* while 100 ng/µl, 200 ng/µl, 500 ng/µl, indicate the concentrations of dsRNA).

3rd instar larvae, then the expression declined to a relatively low level in the 5th instar larvae and 0 h pupae with a transient rise 2 h later, followed a decline to a very low level in the 24 h pupa.

RNAi qRT-PCR down-regulation. Analysis of gene expression after dsRNA injection into *S. exigua* early 5th instar larvae showed significant suppression of target transcripts in the dsRNA-injected larvae compared to the control group three genes tested with three different concentrations (*CPG316, CPG860*, and *CPG4855*). *CPG316* were suppressed with all concentrations of the dsRNA which are 5%, 7.1%, and 8.6% for 100 ng/µl, 200 ng/µl, and 500 ng/µl, respectively (Fig. S2A). In contrast, all concentration of *CPG860* dsRNA was significantly suppressive which were 23.89%, 28.94% and highly suppressed at the highest concentration which is 31.0% (Fig. S2B). In the

case of *CPG4855*, the three concentrations produced 18.2%, 26.17%, and 28.54% suppression. All concentrations are significantly different as compared to the control (Fig. S2C).

Phenotypes of *S. exigua* after treatment with dsRNA. Phenotypic changes were observed in treated larvae compared to controls seven days after the treatment. *CPG316* RNAi retarded growth and interfered with development. After injection of 100 ng/µl, the larvae developed to pupae but could not get ecdysis (Fig. 4). At the higher concentration of 200 ng/µl, some appeared to progress to the prepupal stage, but the larval cuticle became hard and black, and none ecdysis (Fig. 4) with 500 ng/µl RNAi, they all died in half larval and half pupae with hard black cuticle (Fig. 4) *CPG860* RNAi in both the 100 and 200 ng/µl concentrations caused black and hard cuticle on the tergites with some apparently forming prepupae, but do not get ecdysis (Fig. 4). With the highest concentration of dsRNA, the cuticle became completely black and hard and the larvae died (Fig. 4). After injection of 100 ng/µl and 200 ng/ul of *CPG4855* RNAi, larvae eclosed pupal stages but it becomes completely black and dead in the same stage (Fig. 4). At the higher doses, they died as half larvae and half pupal stage (Fig. 4).

Percent mortality. While checking functional analysis through direct injection of dsRNA concentration wise 100, 200, and 500 ng/µl, respectively and dsRed 500 ng/µl as a control. Interference with all three cuticle genes caused high mortality as compared to control (Table S2).

Discussion

In insects, there are various kinds of cuticle-related proteins, which differ in features and numbers in different species. These differences are due to numerous cuticular proteins and the sequence variation among them[12]. Cuticular proteins in a majority of insect species contain the Rebers and Riddiford consensus which is an extended form of a chitin binding region (R&R Consensus)[8,40,41]. Proteins with the Rebers and Riddiford consensus are divided into three classes, RR-1, RR-2, and RR-3 which have some association to the type of cuticle and its regions. There are more cuticular protein motifs reported in *Drosophila melanogaster* such as the Tweedle motif which was found in a protein that controls the larval body shape[9]. Chitin is a major biological polymer[42,43] and its main role is a structural function in arthropod cuticles. Chitin interacts with cuticular protein to form an extremely regular structure. The chitin-binding domain in arthropod peritrophic membrane and chitinase proteins has been characterized and shown to bind chitin[43].

In the current study, we have isolated three novel cuticular protein genes (*CPG316*, *CPG860*, and *CPG4855*) from *S. exigua* pupal integument. The alignment of the deduced amino acid sequences of our genes with those of other species[44] shows identity and similarity in conserved domains. Phylogenetic analysis shows that they are most closely related to cuticular proteins that contain the RR-2 sequence[24,45]. This consensus sequence has been shown to have chitin-binding properties[40].

Genes encoding cuticular proteins are thought to be good models to study the molecular mechanisms of signaling by ecdysteroids and juvenile hormones[46]. We have analyzed the expression pattern of all three isolated genes in different larval stages and at pupal time points. According to qRT-PCR results, *CPG316* mRNA was expressed in both 4th and 5th larval and pupal stages although at ten-fold lower levels than the other two genes. *CPG316* was highly expressed in the 2 h pupal stage and at very low levels in 3rd instar larvae, whereas *CPG860* and *CPG4855* mRNAs were highly expressed in 3rd instar larvae, then fell to low levels in 5th instar larvae with a transient rise in the 2 h pupa. All three genes were expressed at very low levels in the 24 h pupa. Thus, it is likely that they are all contributing to the endocuticle of both larvae and pupae, although *CPG316* may play a somewhat different role from the other two.

In many organisms, to check the functional characterization of a gene, RNA interference (RNAi) has emerged as an influential tool. Use of RNAi shows major promise in biotechnology. Related applications consist of the capacity to avoid unnecessary transgene silencing in genetically engineered lines and the exploitation of various types of silencing to inactivate unwanted genes[47,48]. There are many techniques to introduce dsRNA but direct microinjection is the most general method for delivery of double-stranded RNA (dsRNA) into organisms. The knockdown ability of individual gene selectively through this reverse genetic technique has allowed many scientists to quickly expose the biological function of many genes inside many organisms by causing loss of specific function phenotypes[49]. RNA interference has proved its usefulness in functional genomic research on insects, but it also has considerable potential for the control of pest insects[33].

Our studies using dsRNAs for the three isolated cuticle genes in final instar larvae showed that each suppressed the expression of the particular genes concentration wise significantly suppressed according to qRT-PCR. Yet all three different concentrations produced specific cuticular abnormalities which differed for the three genes. In all concentration of dsRNA, *CPG316* RNAi caused death in the pharate pupal stage. In contrast, *CPG860* RNAi caused the appearance of hard black cuticle on the tergites and also on the intersegmental membranes. These animals died before molting. *CPG4855* dsRNA caused complete cuticle blackness and hardening like pupae. Therefore, later on, these two genes apparently are involved in maintaining flexibility of the larval cuticle whereas *CPG316* may play a role in pupal cuticle formation that is needed for normal ecdysis.

In current research work, three new genes were cloned. All new genes have R&R consensus amino-acids and belong to the RR-2 consensus group. These genes are expressed in the larvae and early pupae but in different patterns. In contrast, Functional analysis results show that it effects on the cuticle in both larval and pupal stages, and necessary for cuticle development, flexibility, and metamorphosis. Further studies are necessary to determine whether these particular cuticular proteins have any role in tissues other than the integument.

References

1. Ali, M., Mishra, B., Polan, M., Ninagi, O. & Swapon, A. Regulation Studies of a Cuticle Protein Underlying Genomic Analysis. *Int J Mol Genet Gene Ther* **1** (2016).
2. Andersen, S. O., Hojrup, P. & Roepstorff, P. Insect cuticular proteins. *Insect biochemistry and molecular biology* **25**, 153–176 (1995).
3. Andersen, S. Cuticular sclerotization and tanning. *Comprehensive Insect Physiology, Biochemistry and Molecular Biology* **4**, 145–170 (2005).
4. Chapman, R. F. *The insects: structure and function*. (Cambridge university press, 1998).
5. Liang, J., Zhang, L., Xiang, Z. & He, N. Expression profile of cuticular genes of silkworm, Bombyx mori. *BMC genomics* **11**, 173 (2010).
6. Fretter, V. & Graham, A. *The functional anatomy of invertebrates*. (Academic press, 1976).
7. Rebers, J. E. & Riddiford, L. M. Structure and expression of a Manduca sexta larval cuticle gene homologous to Drosophila cuticle genes. *Journal of molecular biology* **203**, 411–423 (1988).
8. Togawa, T., Dunn, W. A., Emmons, A. C. & Willis, J. H. CPF and CPFL, two related gene families encoding cuticular proteins of Anopheles gambiae and other insects. *Insect biochemistry and molecular biology* **37**, 675–688 (2007).
9. Guan, X., Middlebrooks, B. W., Alexander, S. & Wasserman, S. A. Mutation of TweedleD, a member of an unconventional cuticle protein family, alters body shape in Drosophila. *Proceedings of the National Academy of Sciences* **103**, 16794–16799 (2006).
10. Cornman, R. S. *et al*. Annotation and analysis of a large cuticular protein family with the R&R Consensus in Anopheles gambiae. *BMC genomics* **9**, 22 (2008).
11. Karouzou, M. V. *et al*. Drosophila cuticular proteins with the R&R Consensus: annotation and classification with a new tool for discriminating RR-1 and RR-2 sequences. *Insect biochemistry and molecular biology* **37**, 754–760 (2007).
12. Futahashi, R. *et al*. Genome-wide identification of cuticular protein genes in the silkworm, Bombyx mori. *Insect biochemistry and molecular biology* **38**, 1138–1146 (2008).
13. Weinstock, G. M. *et al*. Insights into social insects from the genome of the honeybee Apis mellifera. *Nature* **443**, 931–949 (2006).
14. Xiu, W.-M. & Dong, S.-L. Molecular characterization of two pheromone binding proteins and quantitative analysis of their expression in the beet armyworm, Spodoptera exigua Hübner. *Journal of chemical ecology* **33**, 947–961 (2007).
15. Horodyski, F. M. & Riddiford, L. M. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. *Developmental biology* **132**, 292–303 (1989).
16. Apple, R. & Fristrom, J. W. 20-Hydroxyecdysone is required for, and negatively regulates, transcription of Drosophila pupal cuticle protein genes. *Developmental biology* **146**, 569–582 (1991).
17. Hiruma, K., Hardie, J. & Riddiford, L. M. Hormonal regulation of epidermal metamorphosis *in vitro*: control of expression of a larval-specific cuticle gene. *Developmental biology* **144**, 369–378 (1991).
18. Braquart, C., Bouhin, H., Quennedey, A. & Delachambre, J. Up-Regulation of an Adult Cuticular Gene by 20-Hydroxyecdysone in Insect Metamorphosing Epidermis Cultured *in vitro*. *The FEBS Journal* **240**, 336–341 (1996).
19. Shiomi, K., Niimi, T., Imai, K. & Yamashita, O. Structure of the VAP–peptide (BmACP–6.7) gene in the silkworm, Bombyx mori and a possible regulation of its expression by BmFTZ–F1. *Insect biochemistry and molecular biology* **30**, 119–125 (2000).
20. Suzuki, Y., Matsuoka, T., Iimura, Y. & Fujiwara, H. Ecdysteroid-dependent expression of a novel cuticle protein gene BMCPG1 in the silkworm, Bombyx mori. *Insect biochemistry and molecular biology* **32**, 599–607 (2002).
21. Meinke, L. J. & Ware, G. W. Tolerance of three beet armyworm strains in Arizona to methomyl. *Journal of Economic Entomology* **71**, 645–646 (1978).
22. Chaufaux, J. & Ferron, P. Spodoptera exigua Hüb. (Lépid., Noctuidae). *virus* **150**, 50 (1986).
23. Delorme, R. *et al*. Esterase metabolism and reduced penetration are causes of resistance to deltamethrin in Spodoptera exigua HUB (Noctuidea; lepidoptera). *Pesticide Biochemistry and Physiology* **32**, 240–246 (1988).
24. Brewer, M. J. & Trumble, J. T. Field monitoring for insecticide resistance in beet armyworm (Lepidoptera: Noctuidae). *Journal of economic entomology* **82**, 1520–1526 (1989).
25. Brewer, M. J. & Trumble, J. T. Beet armyworm resistance to fenvalerate and methomyl: resistance variation and insecticide synergism. *J. Agric. Entomol* **11**, 291–300 (1994).
26. Van Laecke, K. & Degheele, D. Detoxification of diflubenzuron and teflubenzuron in the larvae of the beet armyworm (Spodoptera exigua) (Lepidoptera: Noctuidae). *Pesticide Biochemistry and Physiology* **40**, 181–190 (1991).
27. Layton, M. in *Beltwide Cotton Conferences (USA)*.
28. East, D., Edelson, J. & Cartwright, B. Relative cabbage consumption by the cabbage looper (Lepidoptera: Noctuidae), beet armyworm (Lepidoptera: Noctuidae), and diamondback moth (Lepidoptera: Plutellidae). *Journal of economic entomology* **82**, 1367–1369 (1989).
29. Liu, S., Wang, M. & Li, X. Pupal melanization is associated with higher fitness in Spodoptera exigua. *Scientific reports* **5** (2015).
30. Ferré, J. & Van Rie, J. Biochemistry and Genetics of Insect Resistance to B acillus thuringiensis. *Annual review of entomology* **47**, 501–533 (2002).
31. Baum, J. A. *et al*. Control of coleopteran insect pests through RNA interference. *Nature biotechnology* **25**, 1322–1326 (2007).
32. Lipardi, C., Wei, Q. & Paterson, B. M. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* **107**, 297–307 (2001).
33. Huvenne, H. & Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *Journal of insect physiology* **56**, 227–235 (2010).
34. Terenius, O. *et al*. RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *Journal of insect physiology* **57**, 231–245 (2011).
35. Fire, A. *et al*. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *nature* **391**, 806–811 (1998).
36. Geley, S. & Müller, C. RNAi: ancient mechanism with a promising future. *Experimental gerontology* **39**, 985–998 (2004).
37. Bell, R. & Joachim, F. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Annals of the Entomological Society of America* **69**, 365–373 (1976).
38. Liu, S., Wang, M. & Li, X. Overexpression of Tyrosine hydroxylase and Dopa decarboxylase associated with pupal melanization in Spodoptera exigua. *Scientific reports* **5** (2015).
39. Crava, C. M., Jakubowska, A. K., Escriche, B., Herrero, S. & Bel, Y. Dissimilar regulation of antimicrobial proteins in the midgut of Spodoptera exigua larvae Challenged with Bacillus thuringiensis toxins or baculovirus. *PloS one* **10**, e0125991 (2015).
40. Rebers, J. E. & Willis, J. H. A conserved domain in arthropod cuticular proteins binds chitin. *Insect biochemistry and molecular biology* **31**, 1083–1093 (2001).
41. Iconomidou, V. A., Willis, J. H. & Hamodrakas, S. J. Unique features of the structural model of 'hard'cuticle proteins: implications for chitin–protein interactions and cross-linking in cuticle. *Insect biochemistry and molecular biology* **35**, 553–560 (2005).
42. Gooday, G. W. in *Advances in microbial ecology* 387–430 (Springer, 1990).
43. Schnellmann, J., Zeltins, A., Blaak, H. & Schrempf, H. The novel lectin-like protein CHB1 is encoded by a chitin-inducible Streptomyces olivaceoviridis gene and binds specifically to crystalline α-chitin of fungi and other organisms. *Molecular microbiology* **13**, 807–819 (1994).
44. Jiang, H., Wang, Y., Korochkina, S. E., Beneš, H. & Kanost, M. R. Molecular cloning of cDNAs for two pro-phenol oxidase subunits from the malaria vector, Anopheles gambiae. *Insect biochemistry and molecular biology* **27**, 693–699 (1997).

45. Zhu, J.-Y., Zhang, L.-F., Ze, S.-Z., Wang, D.-W. & Yang, B. Identification and tissue distribution of odorant binding protein genes in the beet armyworm, Spodoptera exigua. *Journal of insect physiology* **59**, 722–728 (2013).
46. Charles, J.-P. The regulation of expression of insect cuticle protein genes. *Insect biochemistry and molecular biology* **40**, 205–213 (2010).
47. Kooter, J. M., Matzke, M. A. & Meyer, P. Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends in plant science* **4**, 340–347 (1999).
48. Gordon, K. H. & Waterhouse, P. M. RNAi for insect-proof plants. *Nature biotechnology* **25**, 1231–1233 (2007).
49. Regna, K. *et al*. RNAi Trigger Delivery into Anopheles gambiae Pupae. *Journal of visualized experiments: JoVE* (2016).

Acknowledgements

We are very thankful to Lynn M Riddiford of Howard Hughes Medical Institute Ashburn, Virginia, the United States, for English language editing and technical mistake correction in our research article. This work was supported by the National Natural Science Foundation of China (Grant 31171874) and China Scholarship Council Scholarship to Saad Jan.

Author Contributions

Mo Wang, Sisi Liu, and Saad Jan conceived and designed the experiments. Saad Jan and Sisi Liu performed the experiments. Saad Jan and Farman Dawar analyzed the data Jiyun Guo, Chao Gao. Saad Jan, Sisi Liu, Xiangmei Zhang and Muhammad Hafeez wrote the manuscript. Mo Wang and Sisi Liu are corresponding authors. All authors have read and approved the manuscript for publication.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-16435-w.

Competing Interests: The authors declare that they have no competing interests.

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