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RESEARCH PAPER

Knocking down *chitin synthase 2* by RNAi is lethal to the cotton boll weevil

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Abstract The cotton boll weevil (*Anthonomus grandis*) is the most destructive cotton insect pest in Brazil. The endophytic habit of this insect makes difficult its chemical control. Chitin synthase (CHS) is an integral membrane glycosyltransferase that is essential for chitin chain polymerization and deposition in insect chitinous structures, such as the peritrophic membrane (PM). Because it is not present in plants or vertebrates, CHS can be considered a promising target for eco-friendly biotechnological approaches, such as RNA interference (RNAi)-mediated gene silencing. Considering the relevance of *CHS* genes in the chitin biosynthetic pathway in insects, we report here the molecular cloning of the full-length *CHS2* cDNA from the cotton boll weevil, and its functional validation *via* RNAi. The *AgraCHS2* cDNA sequence is 4,869 bp, with a 4,446 bp open reading frame that encodes a predicted protein with 1,482 amino acid residues. Predicted protein has high similarity (53 to 78%) with other insects CHS. Moreover, only one copy is present in *A. grandis* genome. Transcriptional analysis showed that *AgraCHS2* transcripts are restricted to the insect midgut at the third-larval instar and adult stages, which are considered the main feeding stages. RNAi-mediated knockdown of the *AgraCHS2* affected *A. grandis* development, resulting in oviposition reduction of 93% and leading to 100% adult mortality. These data, in addition to the observation of PM severe disorganization in the midgut after *AgraCHS2* knock-down, suggest *AgraCHS2* as a promising target for developing RNAi-based biotechnological alternatives to specifically control the cotton boll weevil.
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Introduction

The gut represents the main barrier between insects and the environment (Terra & Ferreira, 1994; Terra, 2001), and is the major organ involved in food digestion and detoxification of xenobiotics, such as insecticides. Due to its significance, several transcriptomes from insect gut have been sequenced to better understand the molecular features involved in digestion and physiology (Ribeiro et al., 2014; Spit et al., 2016; Swevers et al., 2013; Valencia et al., 2016; Vyas et al., 2017). Thus, a better understanding of the insect gut physiology may contribute to the development of new biotechnological control strategies, such as RNA interference (RNAi) technology (Joga, Zotti, Smagghe, & Christiaens, 2016; Katoch, Sethi, Thakur, & Murdock, 2013). Moreover, the gut is the first contact surface and a direct target of orally delivered RNAi (Shen et al., 2013). Due to the vital importance of the gut in insect development, several studies have reported midgut genes involved in essential metabolic pathways, including the chitin biosynthesis as a target for RNAi-based control (Alvarenga et al., 2016; Kelkenberg, Odman-Naresh, Muthukrishnan, & Merzendorfer, 2015).

Chitin, a linear β-1,4-linked polymer of N-acetylglucosamine, is a versatile biopolymer used by chitin-containing organisms for various anatomical structures (Cohen, 2001). In insects, chitin is found in the cuticle, where it and plays an important role in the formation of body shape and helps protect insects from external attacks (Kramer & Koga, 1986; Moussian, 2010). Chitin is also an important structural component of internal structures such as the trachea cuticle and the gut peritrophic membrane (PM) (Merzendorfer, 2006; Moussian et al., 2015).

Chitin chains are synthesized by membrane-integral glycosyltransferases or chitin synthases (CHSs) (Muthukrishnan, Merzendorfer, Arakane, & Kramer, 2012). Chitin synthase genes have been cloned and characterized in several insects from different orders, including Lepidoptera (Ampasala et al., 2011; Bolognesi et al., 2005; Zhuo et al., 2014), Coleoptera (Alves et al., 2010), Diptera (Gagou, Kapsetaki, Turberg, & Kafetzopoulos, 2002; Arakane et al., 2004; Chen et al., 2013; Zhang, Zhang, Park, & Zhu, 2012), Orthoptera (Alvarenga et al., 2016; Liu et al., 2012), and Hemiptera (Wang et al., 2012; Shang et al., 2016).

In insects, chitin synthase enzymes are classified into two groups, namely, chitin synthase 1 (CHS1) and chitin synthase 2 (CHS2), based on domain composition, sequence homology, tissue localization and physiological role (Merzendorfer, 2011). CHS1 is responsible for chitin synthesis in external and tracheal cuticles as well as in the lining of the fore- and hindgut. CHS2 is responsible for PM chitin synthesis in the midgut (Arakane et al., 2008; Gagou et al., 2002; Hogenkamp et al., 2005). Modification of insect chitin structures can be induced by treatment with synthetic chitin synthesis inhibitors or chitinases, leading to severe physiological dysfunction and insect death (Merzendorfer, 2012; Shang et al., 2016). Functional analyses of insect *CHS1* and *CHS2* genes using RNA interference-mediated (RNAi-mediated) gene silencing have been performed in several insects orders, confirming their specialized functions required for survival, fecundity and egg hatching (Arakane et al., 2008; Firmino et al., 2013; Mansur et al., 2014; Qu & Yang, 2012; Wang et al., 2012; Zhang, Liu, et al., 2010; Zhang, Zhang, & Zhu, 2010).

As chitin is present in the cuticle and PM of insects but is absent in plants and higher-order animals, enzymes participating in chitin biosynthesis, such as CHS, are promising targets for the design of novel strategies for controlling insect pests (Arakane, Taira, Ohnuma, & Fukamizo, 2012).

The PM is an essential multifunctional structure in the insect body where chitin deposition occurs (Kelkenberg et al., 2015; Tellam & Eisemann, 2000), and the PM is closely involved with the digestive process. Furthermore, the PM is a front line of defense against ingested pathogens. Consequently, it is a potential target for the development of novel methods for controlling insect pests. Strategies that aim to modify the structure of the PM generally lead to deficiency of nutrient assimilation or disruption of the digestive process, thereby facilitating pathogenic invasion of insect tissues (Wang & Granados, 2000; Wu et al., 2016).

Similarly, it has been shown that silencing enzymes in the PM chitin synthesis pathway by RNAi has great potential as an insecticidal strategy. Indeed, previous studies have demonstrated that *CHS2* gene silencing in *Diabrotica virgifera* (Coleoptera: Chrysomelidae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) resulted in severe disorders in nutrient assimilation as a consequence of a malformed PM, which resulted in insect death (Alves et al., 2010; Arakane et al., 2008, 2011).

Another coleopteran of major concern is the cotton boll weevil, *Anthonomus grandis* (Boheman, 1843) (Coleoptera: Curculionidae), a major destructive insect pest of cultivated cotton, *Gossypium hirsutum* L., in the tropics. Female adults feed on and lay eggs in cotton flower buds and fruits (IMAmt, 2015). After hatching, the larvae continue to feed on the plant's reproductive structures, which provide them a protective habitat to complete their life cycle. The endophytic behavior of *A. grandis* larvae reduces the effectiveness of chemical control.

Since the mid-1990s, several insect-resistant genetically modified (GM) cotton events expressing *Bacillus thuringiensis* (Bt) entomotoxins have been commercially used throughout the world to control various insect pests. Most of these GM cotton events control lepidopteran pests, increasing crop productivity and decreasing production costs, human intoxication, and environmental contamination by allowing reduced use of chemical pesticides (Klumper & Qaim, 2014). Because none of the commercially available GM cotton events are effective against the cotton boll weevil, it is urgently necessary to develop new tools for controlling this serious insect pest. Genetic silencing *via* RNAi represents one of the most promising technologies with a high biotechnological application potential for controlling coleopteran insect pests, such as the cotton boll weevil (Coelho et al., 2016; Firmino et al., 2013).

Chitin metabolism in *A. grandis* digestive system and its importance in digestion physiology have not been described. Thus, the focus of this study was to determine the importance of chitin synthesis in the *A. grandis* PM. Herein, we have cloned and characterized the *A. grandis* CHS2 (*AgraCHS2*) gene, analyzed the *AgraCHS2* copy number in the *A. grandis* genome and the transcript expression pattern in different life stages and tissues. Moreover, we

Table 1 List of primers used for cloning of *AgraCHS2* cDNA.

Number/primer name	Fragment number	Size (bp)	Primer		
			Direction	Type	Sequence (5′–3′)
1 – NV-d(T)30-AP			A	D	GAATTCACGCGTCGACTAGTAGCATATGTAC(T)30VN
2 – CHSFor1-DPD		1268	S	D	GAYCCNGAYTAYTAYGARTTYGAR
3 – CHSRev1-LHP			A	D	YTCYTGNGGRTGNA
4 – CHSFor2-DGD	1	324	S	D	GAYGGNGAYATYGAYTTY
5 – CHSRev2-QYD			A	D	CCANCKRTCYTCNCCYTGRTCRTAYTG
6 – 5′-1AntgCHS2fw	2	2601	S	E	CAAATTTCTCAAAAATCGCCACC
7 – AntgCHS2cons_rv			A	E	AACATGAGAAATATCGTTCC
8 – 5′-2AntgCHS2fw	3	731	S	E	GGGATTTTGAAATCATACTTGGTA
9 – AntgCHS2-c8194-Rv			A	E	GCGAGCATCAAAAACCATATCC
10 – AgQSconservfw	4	2450	S	E	CGGGGATATTGATTTCCAACC
11 – GTW-AP			A	E	TCCGAATTCACGCGTCGACTAGTAGCA

demonstrated that RNAi-mediated gene silencing of *AgraCHS2* strongly interferes with *A. grandis* development, life-span, and reproduction. Furthermore, we describe malformation of the PM due to *AgraCHS2* silencing. The data obtained here suggest a promising application of *AgraCHS2* knock-down for the biotechnological control of cotton boll weevil.

Material and methods

Insect rearing

A population of *A. grandis* were obtained from the colony maintained at Embrapa Genetic Resources and Biotechnology, Brasília, Brazil. The insects were reared under controlled temperature (27 °C ± 2 °C), relative humidity (70 ± 10%) and light (14 h photoperiod), and were fed daily with standard rearing diet (Monnerat et al., 2000).

Morphological characterization and pH of *A. grandis* gut

Anthonomus grandis eggs were transferred to cotton flower buds, through a small perforation sealed with vaseline, in order to develop and hatch. Third-instar larvae and adults fed with flower buds were recovered and dissected in chilled 150 mM NaCl. The midgut was isolated and divided into the anterior midgut and the posterior midgut. Both midgut samples were separated into tissue and contents. The presence of PM in different sections of the midgut was evaluated as described by Terra (2001). Midgut section contents were washed in 150 mM NaCl and then supplemented with a 5-fold dilution of a universal pH indicator (Merck, pH 4–10). The resulting colored solutions were compared with reference standards. The pH was also measured using Universal pH indicator paper strips (Merck, pH 1–10).

Cloning and sequencing of the *AgraCHS2* gene

Total RNA was isolated from dissected midguts of third-instar larvae using Trizol reagent (Invitrogen, USA). The Superscript II Kit TM First-Strand Synthesis System for RT-PCR (Invitrogen, USA) was used to prepare cDNA according to the manufacturer's instructions. Pairs of gene-specific and degenerate primers, designed from conserved regions of insect CHSs and paralogous contig sequences found in the *A. grandis* transcriptome (Firmino et al., 2013), were used to obtain overlapping PCR products using cDNA as the template. The complete sequence of *AgraCHS2* was obtained using 5′- and 3′-RACE. The sequences and properties of the primers used are presented in Table 1. All PCR products were cloned into a pGEM-T easy vector (Promega, USA) and transformed into *E. coli* OmniMAX™ electrocompetent cells. Following plasmid DNA purification, the sequence of each construct was determined using an automatic DNA sequencer (ABI3100, Applied Biosystems). The resulting overlapping sequences were assembled, and the remaining sequence gaps of the *AgraCHS2* gene were filled by aligning PCR-derived sequences with *A. grandis* transcriptome paralogous contig sequences (Firmino et al., 2013), which resulted in its complete sequence.

Bioinformatic analysis of the AgraCHS primary protein sequence

The amino acid sequence of AgraCHS2 was predicted following translation of the corresponding cDNA sequence using BioEdit software (Hall, 1999). Transmembrane helices and coiled-coil domains were predicted using TMHMM v.2.0 (Krogh, Larsson, von Heijne, Sonnhammer, 2001) and Paircoil software (McDonnell, Jiang, Keating, & Berger, 2006), respectively. Other protein sequence analysis tools used in this study, including MW and p*I*, were obtained from the ExPASy Proteomics website (http://us.expasy.org/). Putative N-glycosylation sites were found out using NetNGly software, and the prediction of putative O-glycosylation sites was predicted using YinOYang software. Sequence alignments were performed using ClustalW (BLOSUM 62). The phylogenic analysis was conducted in MEGA version 5, applying the bootstrap test with 10,000 replicates to calculate the percentages of replicate trees in which sequences were clustered together (Tamura et al., 2011).

Southern blot analysis of *A. grandis* genomic DNA for *CHS2* sequences

The number of copies of the *AgraCHS2* gene within the *A. grandis* genome was determined by using genomic DNA. Three different restriction enzymes, *Xba*I, *Sph*I, and *Nco*I, were used to digest 8 μg of DNA. The resulting digests were subjected to electrophoresis in 0.9% agarose gel, transferred to a Hybond N+ Nylon membrane (Amersham, UK) under alkaline conditions, and washed as described by Sambrook, Fritsch, and Maniatis (1989). The probe designed corresponded to a 1,348 bp PCR fragment spanning nucleotide positions 1,248 to 2,595 of the *AgraCHS2* cDNA. The probe was radiolabeled with [α-^{32}P] dCTP by using a Ready-To-Go DNA Labeling Beads Kit (GE Healthcare, UK). Unincorporated nucleotides were removed by using MicroSpin S-300 HR columns (Amersham, UK), and the membrane was incubated in hybridization solution (5X SSC, 5 X Denhardt's solution, 0.5% SDS) at 60 °C for 24 h. After hybridization, the membrane was washed 3 times with 1X SSC solution for 15 min and subsequently exposed to an imaging plate (BAS-MP, FujiFilm, Japan) for 24 h. Images were acquired by using an FLA3000 Phosphorimager (FujiFilm, Japan).

Tissue and developmental stage-dependent gene transcription profiles

The epidermis and midgut were dissected in DEPC-treated water from both larvae and adult insects. For developmental stage-dependent transcript profiling, all *A. grandis* stages, including eggs, first-, second-, and third-instar larvae, and adults of both sexes were used. Total RNA was isolated from different tissues and from entire insects at different developmental stages by using Trizol reagent (Thermo Fisher Scientific, USA). For each sample, 1 μg of total RNA treated with Ambion® DNase I RNase-free™ (Thermo Fisher Scientific, USA) was used to synthesize the first-strand cDNA using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, USA). The cDNA prepared from total RNA was used as a template for quantitative real-time PCR (qPCR) and reverse transcription PCR (RT-PCR) analyses. The sequences of the primers used for RT-qPCR analysis were designed using Primer 3 software. GAPDH and β-actin were used as reference genes. The following *AgraCHS2* primers were used: forward 5′ AAGGCATTAACGGTGACGAC 3′ and reverse 5′ TCCAAGTCGTTGATGACTGC 3′. The following β-actin reference gene primers were used: forward 5′ CCTTTAACACCCCTGCTATG 3′ and reverse 5′ TGAGGTAGTCGGTCAAGTCA 3′. The following β-actin reference gene primers were used: forward 5′ CCTTTAACACCCCTGCTATG 3′ and reverse 5′ TGAGGTAGTCGGTCAAGTCA 3′. The qPCR analysis was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and Rox plus SYBR Green Master Mix 2X (LGC). The PCR cycling parameters were 95 °C for 15 min to activate the hot-start Taq DNA polymerase, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s. At the end of each qPCR experiment, a melt curve was generated to evaluate the presence of nonspecific products or primer-dimer formation. The qPCR amplifications were performed in technical triplicates and biological duplicates. Raw fluorescence data for all runs were imported into Real-time PCR Miner software (Zhao & Fernald, 2005) and used to determine the Ct values and PCR efficiency. Analyses of *AgraCHS2* expression were performed by using qBASE Plus software (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007).

Synthesis of dsRNA

A 200 bp fragment from the *AgraCHS2* gene (nucleotide position 4352–4552) and a 400 bp fragment from the *GUS* gene (negative control) were cloned into the pL4440 vector (Timmons, Court, & Fire, 2001) and sequenced. *In vitro* transcription of dsRNA was performed according to the protocol of the MEGAscript High Yield Transcription Kit (Thermo Fisher Scientific, USA), as described by Tomoyasu and Denell (2004). The *AgraCHS2* and *GUS* clones were used as templates for PCR using a T7 primer. After *in vitro* transcription, the resulting synthetic dsRNAs were purified by phenol/chloroform extraction followed by isopropanol precipitation. Precipitated synthetic AgraCHS2 dsRNA and GUS dsRNA samples were solubilized in DEPC-treated water and quantified by spectroscopy, and the integrity was assessed using 1% agarose gel electrophoresis. The final concentrations of synthetic AgraCHS2 dsRNA and GUS dsRNA were adjusted to 200 ng/μL.

Functional analysis of *AgraCHS2* by RNAi-mediated gene knockdown

Knockdown of chitin synthase 2 was performed by microinjecting sequence-specific synthetic *AgraCHS2* dsRNA (or, as negative controls, synthetic GUS dsRNA or water) in *A. grandis*. The dsRNAs were injected into third-instar larvae or adult insects using a 10-μL Hamilton syringe. The insects were anesthetized on ice for 10 min and subsequently microinjected.

In the experiment using larvae, 200 ng dsRNA was injected into the dorsal side of the fourth or fifth abdominal segment of each larva. The microinjected larvae were maintained on an artificial diet. At 72 h post-microinjection, five microinjected larvae were randomly removed from the artificial diet for qPCR analysis of the RNAi-mediated silencing of *AgraCHS2* expression.

To evaluate the silencing of *AgraCHS2* in adult insects, 200 ng of dsRNA was injected into the abdominal cavity under the elytra. The microinjected insects were maintained on an artificial diet. At 72 h post-microinjection, the insects were removed for AgraCHS2 expression analysis.

To evaluate the effect of silencing *AgraCHS2* on *A. grandis* female phenotype parameters, 3-day post-eclosion females were anesthetized and subsequently microinjected with 1 μL of synthetic dsRNA solution (200 ng/μL) or water into the dorsal region of the abdomen under the elytra. In this case, each experimental unit consisted of 16 microinjected female insects and 8 non-microinjected male insects. Following microinjection, the adult insects were kept in cages. The total experimental period comprised 12 days:

the insects were counted every 48 h to assess mortality, and the eggs were counted to evaluate oviposition. The cDNA synthesis, qPCR, primers and reaction conditions were as described above. The qPCR amplifications were performed in technical triplicates and biological duplicates. The data were analyzed by one-way analysis of variance (ANOVA), and means were compared using Tukey's test.

Morphological and histological analysis of *A. grandis* midgut upon RNAi-mediated gene silencing of *AgraCHS2*

A. grandis midguts treated either with synthetic *AgraCHS2* dsRNA or with synthetic *GUS* dsRNA were histologically evaluated in detail, as described by Coelho et al. (2016). Briefly, midguts were dissected at 5 days post-microinjection, fixed in 2.5% glutaraldehyde, gradually dehydrated in ethanol, and finally embedded in Technovit 7100 (Heraeus Kulzer, Germany). Five-micrometer-thick midgut tissue sections were subsequently cut, stained with 0.05% toluidine blue and mounted in DPX (Sigma-Aldrich, USA). Microscopic analyses were performed using bright-field optics, and images were acquired with a digital camera (Axiocam, Zeiss, Germany).

Results

Anthonomus grandis peritrophic membrane is localized only in the posterior midgut

The *A. grandis* alimentary channel is composed of a short foregut, a long midgut, and insertion of the Malpighian tubules followed by the hindgut in both larvae and adult insects (Fig. 1A and D). The foregut begins at the mouth and is formed by a short tube, which leads to the midgut or ventricle (Fig. 1A). The adult foregut presents a proventriculus containing sclerotic dentate plates (Fig. 1E). The midgut is longer in larvae than in adult insects (Table 2). The diameter of the midgut decreases from the anterior to posterior regions, and the posterior midgut is the longest portion of the *A. grandis* gut. Small papilliform projections are observed in the terminal portion of the posterior midgut (Fig. 1C) (Snodgrass, 1993). The hindgut is short, presents a folded ileum and a colon, followed by a rectum that terminates at the anus (Fig. 1A). The pH varies along the *A. grandis* midgut, ranging from 5.0 ± 0.1 in the anterior midgut (Fig. 1B and E) to 7.0 ± 0.1 at the posterior midgut. This pH range is the same for larvae and adults.

The PM was detected in different regions of the midgut based on the method described by Terra (2001). In both larvae and adults, the anterior midgut (Fig. 1B and E) leaks its luminal content when pierced, and the gelatinous material surrounding this content cannot be collected. However, when tearing the posterior midgut (Fig. 1F), there is no leakage of luminal content, and a structured film surrounding the midgut content can be collected. These observations indicate that the midgut lumen contents are surrounded by a peritrophic gel (PG) at the anterior midgut and a PM exclusively localized in the posterior midgut (Terra, 2001) (Fig. 1G).

AgraCHS2 is a putative membrane-integral protein with typical insect CHS conserved domains

The full-length cDNA sequence of *AgraCHS2* was obtained by PCR amplification with degenerated and specific primers using 5′ and 3′ RACE. The cDNA template was prepared from larval midgut total RNA. The sequence (GenBank ID: KF147150.1) consists of 4,832 bp, including an open reading frame (ORF) of 4,446 bp that encodes 1,482 amino acid residues, flanked by 245- and 142-bp untranslated regions (UTRs) at the 5′ and 3′ ends, respectively (Fig. 2A). The *in silico* calculated MW and p*I* of the predicted protein are 169.44 kDa and 6.06, respectively. Fifteen hydrophobic membrane-spanning α-helices were predicted, suggesting that AgraCHS2 is a membrane-integral protein (Fig. 2B). Like other insects CHS, AgraCHS2 has a modular structure (Merzendorfer, 2006); it has an N-terminal domain (domain A) with 8 membrane-spanning helices, a highly conserved central domain (domain B) that contains two motifs (EDR and QRRRW) that are regarded as signature sequences of chitin synthases, and a C-terminal domain (domain C) with an additional 7 transmembrane regions, which are characteristics of glycosyltransferase family 2 enzymes (Fig. 2C). In addition, AgraCHS2 contains six potential N-glycosylation sites and four putative O-glycosylation sites distributed throughout the sequence, suggesting that the protein can be glycosylated (Fig. 2B).

Multiple alignments of the AgraCHS2 catalytic domain sequence with other insects CHS catalytic sequences indicates a high degree of conservation, particularly in amino acid residues involved in substrate binding (Merzendorfer, 2006) (Fig. 3).

AgraCHS2 is less conserved among Coleoptera than is CHS1

The phylogenetic tree of the full-length CHS sequences was generated based on the multiple alignments of the amino acid sequence with other insect CHS sequences. The unrooted tree shows that CHS1 and CHS2 from different species are clearly grouped into two different clusters (Fig. 4). As shown in the two-dimensional protein pairwise identity/similarity plot (Fig. 4), CHS1 is highly conserved among insect orders and presents identity/similarity indexes over 70%. However, CHS2 is more variable among the orders, showing similarity indexes of 30–60%, except for the lepidopteran CHS2, which has similarity indexes over 80%. The deduced amino acid sequence of AgraCHS2 is highly similar (78.8%) and identical (66.5%) to the CHS2 of the coleopteran *Dendroctonus ponderosae* (Fig. 4).

The number of copies of *AgraCHS2* within the genome of *A. grandis* was determined by Southern blotting. A single intense hybridization band was detected in *Xba*I, *Sph*I, and *Nco*I single digestions, meaning that there is only one copy of *AgraCHS2* in the *A. grandis* haploid genome (Fig. 5).

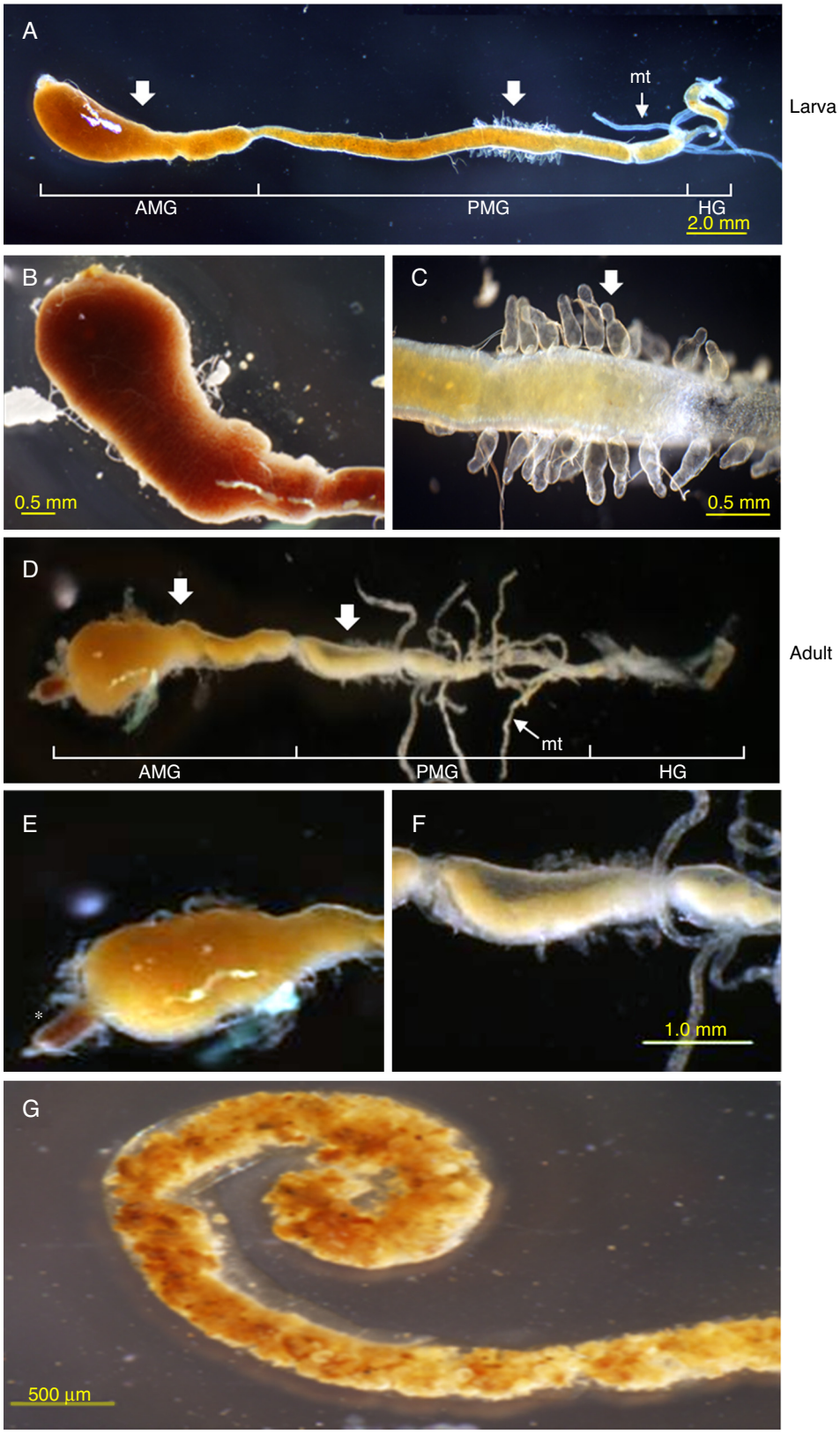

Figure 1 Morphology of the *A. grandis* digestive system. (A) *Anthonomus grandis* larval intestinal system, showing a short foregut, a long midgut, and a short hindgut. Regions indicated by the arrows are enlarged in (B) and (C). (B) Larval anterior midgut; this region presents a pH of 5.0 and no PM. (C) Papilliform projections, found in the PMG region with a pH of 7.0. (D) *Anthonomus grandis* adult intestinal system; regions indicated by the arrows are enlarged in (E) and (F). (E) Adult anterior midgut; the region indicated by the asterisk is the proventriculus. (F) Adult posterior midgut, presenting a PM. (G). Peritrophic membrane. AMG = anterior midgut; PMG = posterior midgut; HG = Hind gut; MT = Malpighian tubules; PM = Peritrophic membrane.

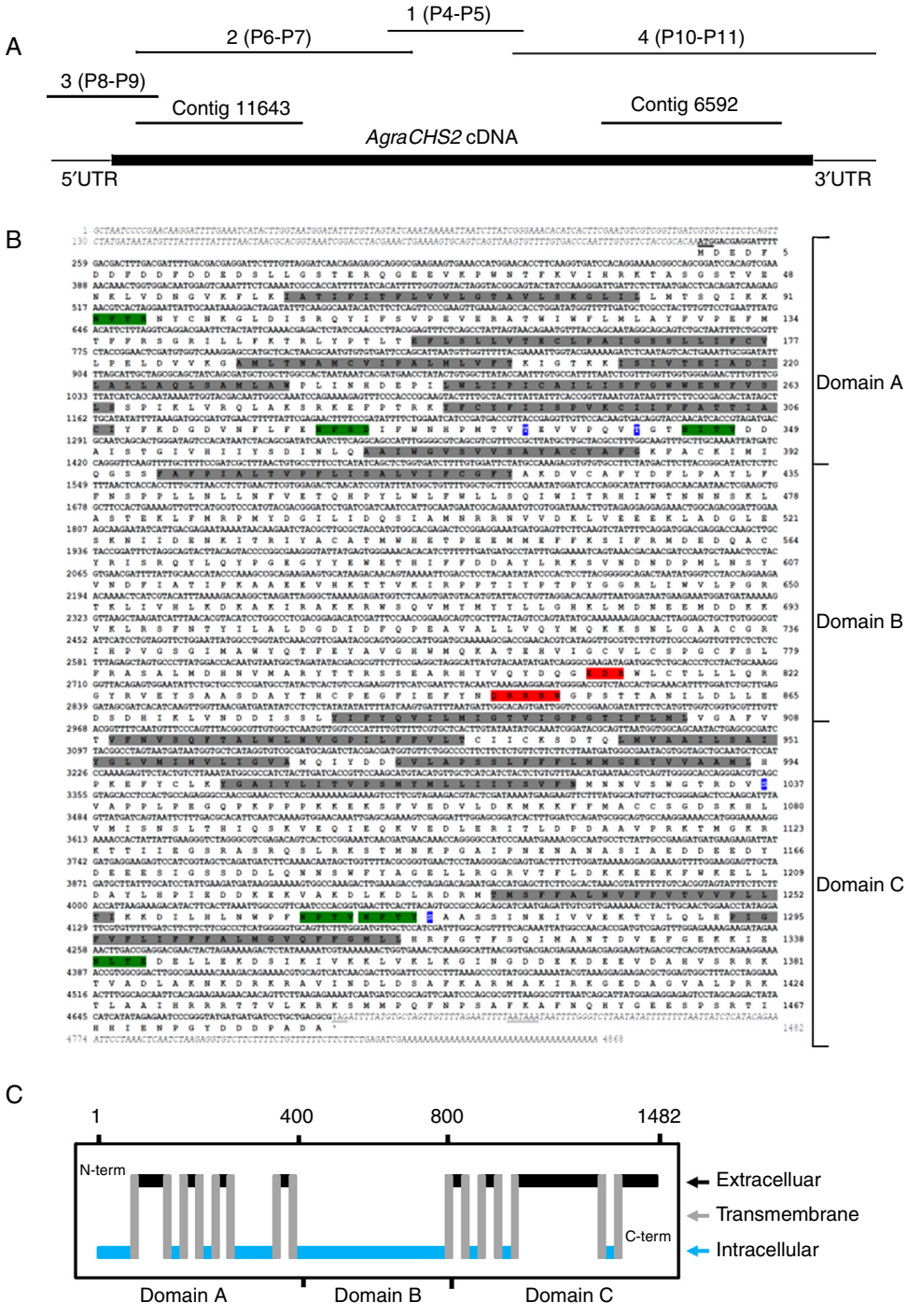

Figure 2 Sequence analysis of the *AgraCHS2* gene. (A) Amplicons and transcriptome contigs obtained that resulted in the full-length sequence of the *AgraCHS2* gene. Amplicons are numbered as indicated in Table 1. (B) Nucleotide and deduced amino acid sequences of AgraCHS2. Numbering on the left refers to the nucleotide sequence while numbering on the right refers to the deduced amino acid sequence. ATG = start codon; TAA = stop codon; AATAAA = putative polyadenylation signal. The 5′UTR and 3′UTR are shown in gray italic characters. Regions corresponding to the A, B and C domains are indicated. Fifteen predicted hydrophobic membrane-spanning α-helices are highlighted in gray. The two chitin synthase signature motifs (EDR and QRRRW) are highlighted in red. Six putative N-glycosylation sites are highlighted in green. Four putative O-glycosylation sites, which were predicted using the YinOYang program, are highlighted in blue. (C) Predicted intracellular, transmembrane and extracellular secondary structures of AgraCHS2. See the text for methodological details.

Table 2 *A. grandis* midgut morphometry.

	Midgut length (mm)	Anterior midgut (mm)		Posterior midgut (mm)		Hindgut (mm)	
		Length	Diameter	Length	Diameter	Length	Diameter
Larvae	33.0 ± 0.4	7.8 ± 0.06	2.5 ± 0.02	25.2 ± 0.05	0.8 ± 0.01	4.0 ± 0.02	0.40 ± 0.02
Adult	10.2 ± 0.1	4.1 ± 0.05	1.5 ± 0.05	6.0 ± 0.04	0.4 ± 0.02	3.1 ± 0.06	0.2 ± 0.01

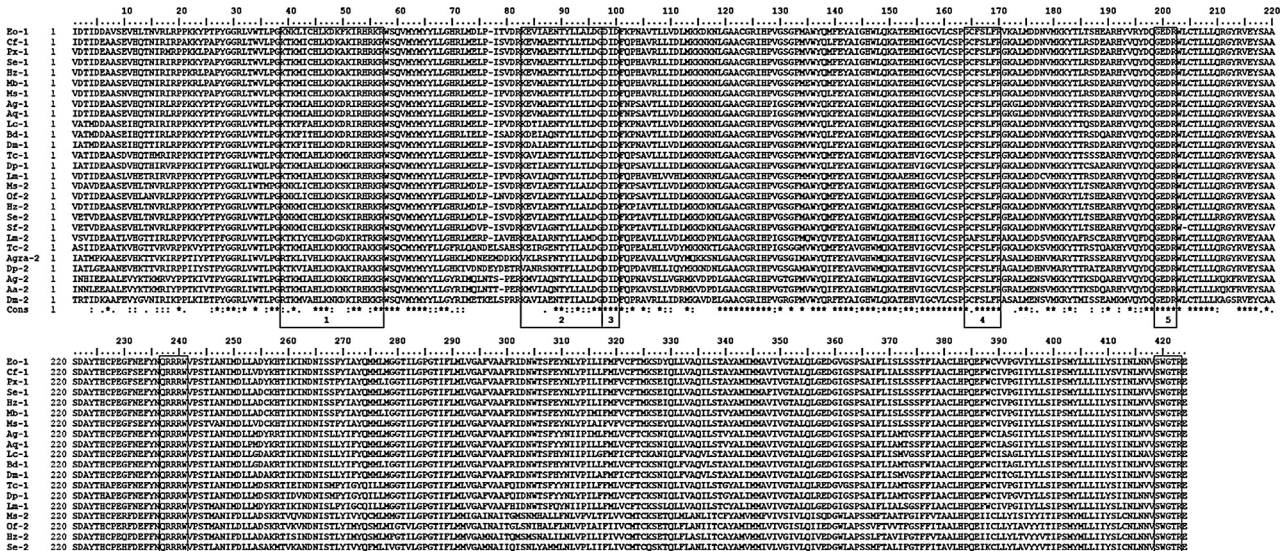

Figure 3 Multiple alignment of *A. grandis* chitin synthase (CHS2) catalytic domain sequence with other insect CHS catalytic domain sequences. For multiple alignment analysis, chitin synthases were included: Agra-2 = *A. grandis* CHS2 GenBank ID KF147150.1 as described in the present work; Aa-2 = *Aedes aegypti* CHS2 GenBank ID AAF34699.2; Ag-1 = *Anopheles gambiae* CHS1 GenBank ID XP_321336.5; Ag-2 = *A. gambiae* CHS2 GenBank ID XP_321951.2; Aq-1 = *Anopheles quadrimaculatus* CHS1 GenBank ID ABD74441.1; Dm-1 = *Drosophila melanogaster* CHS1 GenBank ID NP_730928.2; Dm-2 = *D. melanogaster* CHS2 GenBank ID NP_001137997.2; Bd-1 = *Bactrocera dorsalis* CHS1 GenBank ID AEN03040.1; Eo-1 = *Ectropis obliqua* CHS1 GenBank ID ACA50098.1; Ms-1 = *Manduca sexta* CHS1 GenBank ID AAL38051.2; Ms-2 = *M. sexta* CHS2 GenBank ID AAX20091.1; Of-2 = *Ostrinia furnacalis* CHS2 GenBank ID ABB97082.1; Px1 = *Plutella xylostella* CHS1 GenBank ID BAF47974.1; Se-1 = *S. exigua* CHS1 GenBank ID AAZ03545.1; Se-2 = *S. exigua* CHS2 GenBank ID ABI96087.1; Sf-2 = *S. frugiperda* CHS2 GenBank ID AAS12599.1; Cf-1 = *Choristoneura fumiferana* CHS1 GenBank ID ACD84882.1; Bm-1 = *Bombyx mori* CHS1 GenBank ID NP_001245291.1; Mb-1 = *Mamestra brassicae* CHS1 GenBank ID ABX56676.2; Hz-1 = *Helicoverpa zea* CHS1 GenBank ID 9ADX66429.1; Hz-2 = *H. zea* CHS2 GenBank ID ADX66427.1; Tc-1 = *T. castaneum* CHS1 GenBank ID AAQ55059.1; Tc-2 = *T. castaneum* CHS2 GenBank ID AAQ55061.1; Lm-1 = *Locusta migratoria* CHS1 GenBank ID ACY38588.1; Lm-2 = *L. migratoria* CHS2 GenBank ID AFK08615.1; Ce-2 = *Caenorhabditis elegans* CHS2 GenBank ID AAX62733.1. Symbols in the consensus sequence indicate identity (*), highly conserved substitutions (:), and conserved substitutions (.). Numbered boxes refer to highly conserved regions found in many GTF2 enzymes, as cited by Merzendorfer (2006).

AgraCHS2 is highly transcribed in the midgut during the feeding stages of *A. grandis*

Quantitative PCR experiments were carried out to analyze the transcription pattern of *AgraCHS2* during cotton boll weevil development (Fig. 6A). *AgraCHS2* presented low expression during the egg stage and first and second larval instars. In contrast, it was highly expressed in third-instar larvae and adults of both sexes. A drastic reduction in *AgraCHS2* transcripts during the pupal stage was observed compared with third-instar larvae. Thus, *AgraCHS2* transcript accumulation is dynamic through the various insect developmental stages, being mainly expressed during feeding stages. *AgraCHS2* transcript expression in the midgut or tegument tissues from larvae and adult insects of *A. grandis* was analyzed further (Fig. 6B). In both biological forms, the expression of *AgraCHS2* was found to be restricted to the midgut, with no detectable expression in the tegument tissues, indicating its specificity in digestive organs.

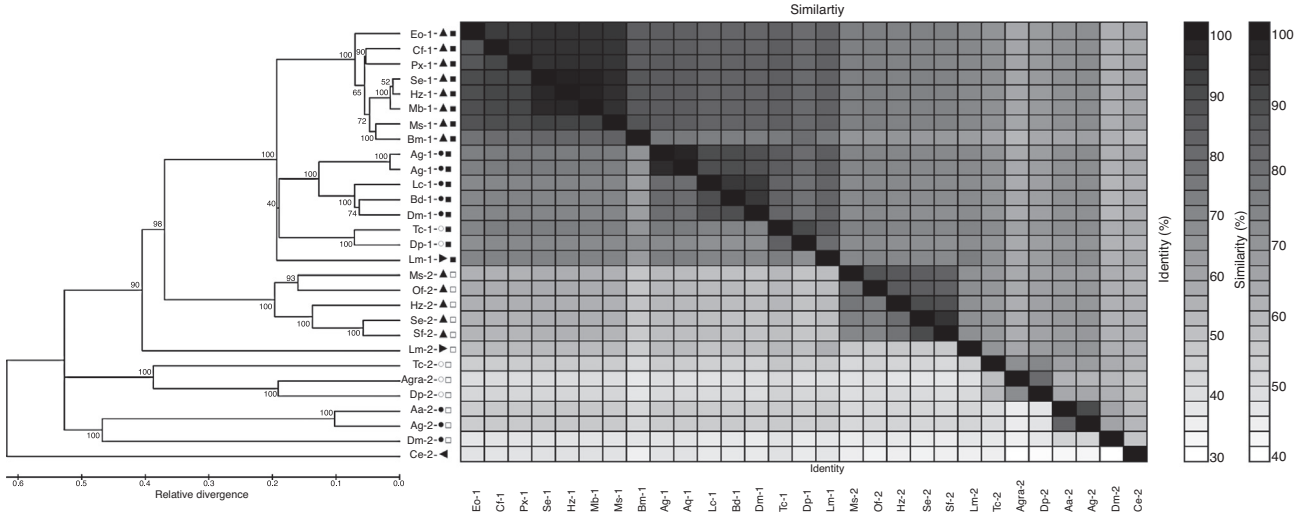

Figure 4 Insect CHS dendrogram and two-dimensional protein pairwise identity/similarity plot illustrating the phylogenetic relationships. The tree was generated by MEGA 5 based on the protein sequences of insect CHS mentioned above. The dendrogram was rooted with *C. elegans*. CHS class 1 or 2, as indicated in each case. Markers indicate insect orders, *i.e.*, Lepidoptera (△), Diptera (●), Orthoptera (►) and Coleoptera (◯), and the presence (■) or absence (□) of a coiled-coil domain. The identity/similarity matrix was generated with protein pairwise alignment values (at the right of the figure). Numbers on the branches represent bootstrap values for 10,000 replicates.

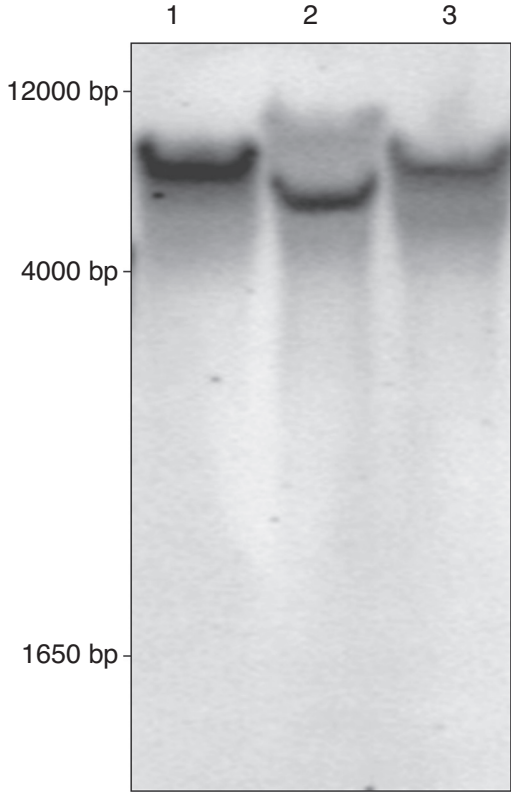

Figure 5 Determination of the number of copies of the *AgraCHS2* gene within the genome of *A. grandis* by Southern blot analysis. Genomic DNA of *A. grandis* (8 μg per line) was digested with four restriction enzymes and separated by electrophoresis as indicated: line 1 (*Xba*I), line 2 (*Sph*I) and line 3 (*Nco*I). The membrane was probed with a 1348-bp DNA fragment amplified from *AgraCHS2* cDNA and labeled with ^{32}P dCTP. DNA size markers are indicated in bp.

RNAi-mediated silencing of *AgraCHS2* reduces the egg production and life-span of *A. grandis*

The biological role of AgraCHS2 during *A. grandis* development was validated by RNAi. *AgraCHS2* dsRNA was synthesized *in vitro* and microinjected into the hemolymph of third-instar larvae and of three-day-old adult females. To evaluate the effectiveness of *AgraCHS2* RNAi-mediated gene silencing, PCR quantification of transcripts *via* qPCR was performed using total RNA extracted from larvae and adult insects at 72 h after microinjection. The target gene *AgraCHS2* was effectively suppressed by RNAi in the third-instar larvae and in adult insects. Levels of *AgraCHS2* transcripts were reduced by 15 and 29 times, respectively, in larvae and adults compared with control insects microinjected with *GUS*-dsRNA (Fig. 7). There was no difference in *AgraCHS2* expression between insects injected with water and those injected with GUS dsRNA. The silencing effect was also evaluated. In this experiment, larvae were microinjected with dsRNA and allowed to develop into adults. The adult insects with *AgraCHS2* silenced *via* dsRNA treatment presented low transcript levels (Fig. 7) and did not survive after 5 days (data not shown).

AgraCHS2-silenced *A. grandis* insects exhibited reduced oviposition and survival. In females microinjected with *AgraCHS2* dsRNA, the oviposition was reduced by 93%, and 100% of insects were dead at ten days post-microinjection (Fig. 8A and B).

Morphological and histological analysis of *AgraCHS2*-silenced *A. grandis* midgut

The midgut morphology of *AgraCHS2*–silenced larvae differed from control insects; *AgraCHS2*–silenced midguts exhibited increased size and brighter and more viscous

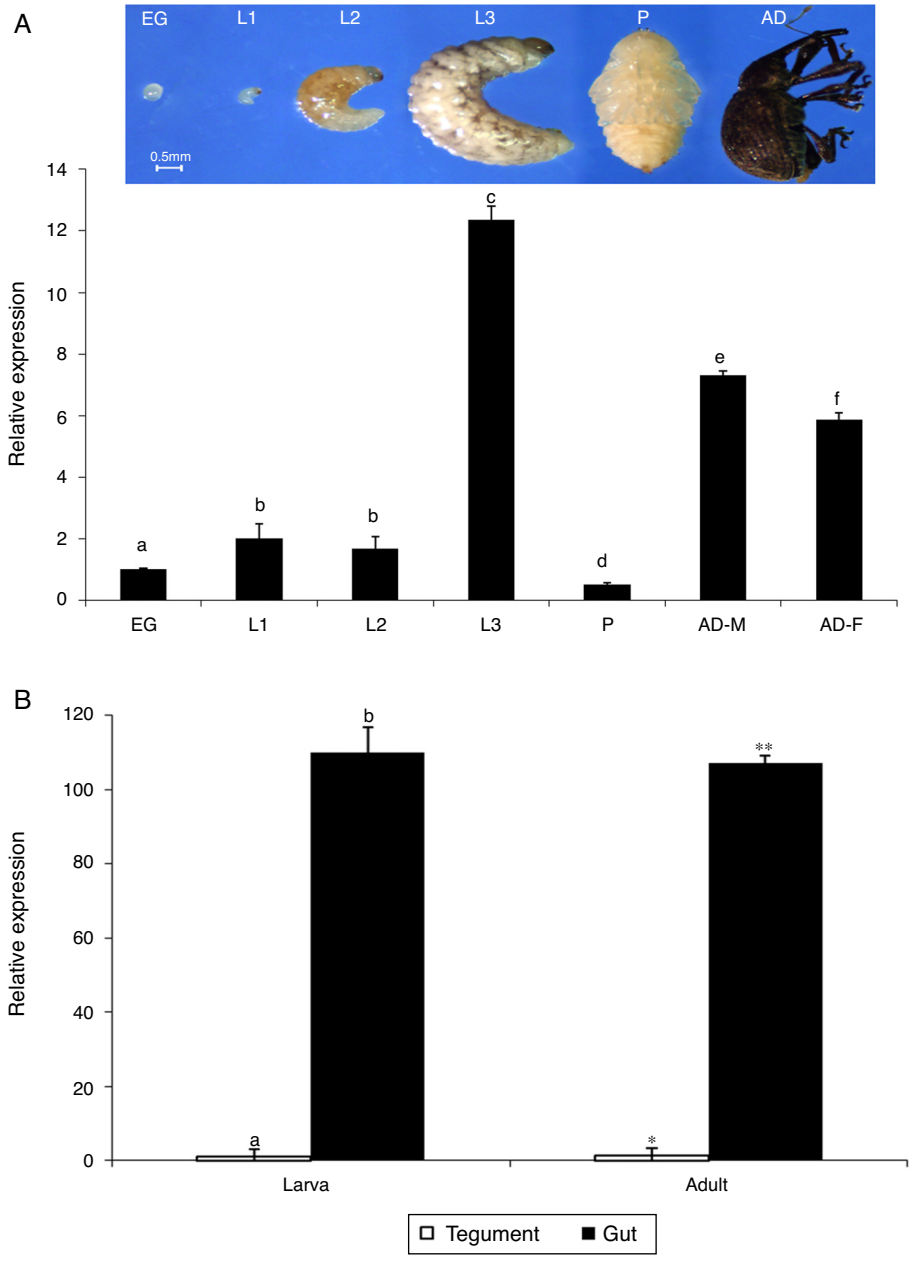

Figure 6 Expression pattern of *AgraCHS2* transcripts in *A. grandis*. (A) AgraCHS2 transcript profile of various developmental stages. Transcript accumulation was determined by qPCR by using cDNA from *A. grandis* at various developmental stages, *i.e.*, eggs (EG), first instar larvae (L1), second instar larvae (L2), third-instar larvae (L3), pupa (P), male adults (AD-M) and female adults (AD-F), as depicted in the upper left corner panel. (B) Relative transcript levels of *AgraCHS2* in midgut and carcass tissues (tegument) from third-instar larvae and adult *A. grandis*. GAPDH and β-actin were used as internal reference genes for qPCR normalization. The expression profile was relative to the egg stage, which was defined as 1. Different letters above the bars indicate statistically significant differences of AgraCHS2 expression among the various *A. grandis* developmental stages based on two biological replications ($P < 0.05$, Tukey's test).

content (Fig. 9A). Histological sections of *AgraCHS2*-silenced *A. grandis* midguts also differed from controls, presenting several areas of cellular disruption, probably due to abrasion by food particles that remained in direct contact with the intestinal epithelium without the protection of the malformed PM (Fig. 9B).

Discussion

There is substantial morphological diversity among structures in the digestive systems of insects, allowing insects the flexibility to adapt to a wide variety of food types. The high digestive efficiency of the insect gut is due to

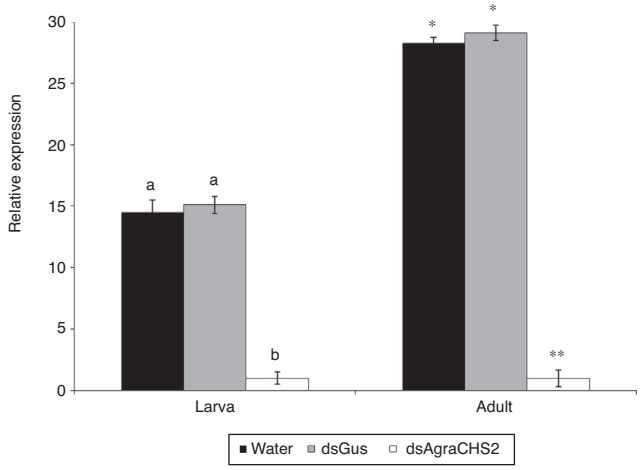

Figure 7 Relative transcript levels of *AgraCHS2* in dsRNA-treated insects. Negative control treatments were performed by microinjection of *GUS* dsRNA or water. *GAPDH* and β-*actin* were used as internal reference genes for normalization of qPCR. The lowest expression value was considered as 1, and the others are reported as relative to the lowest value. Different letters/asterisks above the bars indicate statistically significant differences in expression among different tissues based on three biological replications ($P < 0.05$, *t*-test).

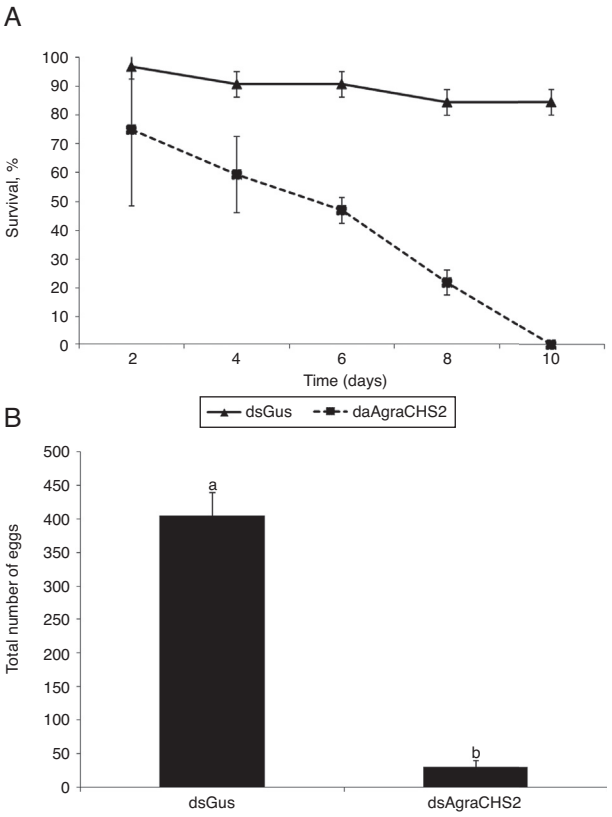

Figure 8 Effects of RNAi-mediated silencing of *AgraCHS2* on adult female *A. grandis* insects. (A) Effect of RNAi-mediated silencing of AgraCHS2 on the survival rate of *A. grandis* females. Adult females were microinjected with *AgraCHS2* dsRNA or negative control *GUS* dsRNA (200 ng per insect). Female insects were then maintained on artificial diet in cages. (B) Number of eggs per female per day; Overall oviposition (accumulative during 10 days). Each data point is the mean of six replicates. Mean values followed by the same letter were not significantly different ($P < 0.05$) by Tukey's test. Error bars represent the standard deviation. Sixteen adult females were microinjected with *AgraCHS2* dsRNA or negative control *GUS* dsRNA (200 ng per insect), followed by mating with untreated males. Female insects were then maintained on an artificial diet in cages. The number of eggs was counted every 48 h.

compartmentalization (Terra & Ferreira, 1994; Terra, 2001). In insects, the gut is divided into three main regions: the foregut, the midgut, and the hindgut. Gut morphology in *A. grandis* is similar to that in other insects belonging to the Curculionidae family (Caldeira, Dias, Terra, & Ribeiro, 2007; Rubio et al., 2008; Sousa, Scudeler, Abrahão, & Conte, 2014). Therefore, the dilated initial portion of the midgut of *A. grandis* adults and larvae has an acidic pH without the PM but with a peritrophic gel (Terra, 2001). The posterior portion of the midgut has a neutral pH, similar to *Hypothenemus hampei* (Coleoptera: Curculionidae), *Sphenophorus levis* (Coleoptera: Curculionidae) and *Dermestes maculatus* (Coleoptera: Dermestidae) (Caldeira et al., 2007; Soares-Costa et al., 2011; Sousa et al., 2014; Valencia, Bustillo, Ossa, & Chrispeels, 2000). In most coleopteran families, the midgut content is usually acidic in the anterior region but near neutral or alkaline in the posterior region (Soares-Costa et al., 2011; Terra & Ferreira, 1994). As demonstrated by Oliveira-Neto et al. (2003), pollen grains rich in starch are found in cotton floral buds, which are the main feeding sites of the cotton boll weevil. Thus, high α-amylase activity in the anterior midgut and high proteolytic activity in the posterior portion is usually associated with this pH gradient (Oliveira-Neto et al., 2003, 2004).

A semipermeable PM allows the presence of a countercurrent flow within the insect gut, termed endoectoperitrophic circulation (Terra, 2001). This flow, from the posterior to the anterior midgut, allows the spatial and temporal separation of digestion and, simultaneously, enables a high rate of nutrient absorption and recycling of digestive enzymes (Kelkenberg et al., 2015; Terra, 2001). Due to the importance of the PM in the insect digestive processes, the use of insect control strategies based on the interruption of metabolic pathways for the synthesis of insect PM components, such as interrupting the synthesis of chitin fibers, is a potential biotechnological approach (Kola, Renuka, Madhav, & Mangrauthia, 2015).

In all chitin-containing organisms, chitin synthesis is directed by the action of a chitin synthase (Merzendorfer, 2011). Numerous insect CHSs have been reported to date from multiple insect species of various orders, including Diptera, Lepidoptera, Coleoptera, Orthoptera, Hemiptera and Hymenoptera (Alvarenga et al., 2016; Alves et al., 2010; Chen et al., 2013; Zhuo et al., 2014). Based on their amino acid sequence similarities and functions, insect CHSs are classified into two groups, CHS1 and CHS2. Most insect genomes encode both CHS1 and CHS2. CHS2 is usually expressed in midgut epithelial cells for chitin synthesis in the PM (Zimoch et al., 2005; Liang et al., 2010). In this study, we describe the cloning and characterization of a cDNA containing the full *A. grandis* CHS2 ORF, named *AgraCHS2*.

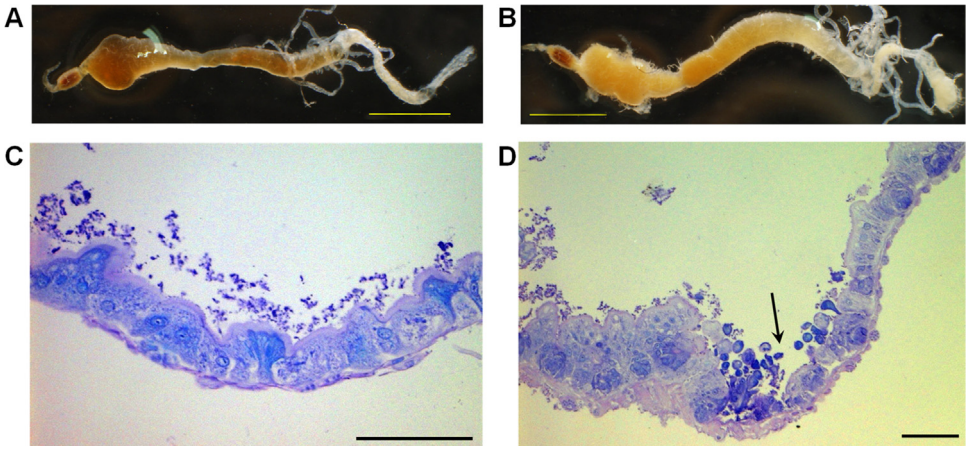

Figure 9 Morphological and histological analysis of the gut from RNAi-mediated *AgraCHS2* silenced *A. grandis*. (A and B) Gut morphology. Guts were dissected 5 days after microinjection of *GUS* dsRNA or *AgraCHS2* dsRNA, as indicated. (C and D) Gut histology. Bright-field images of transversal sections of the gut stained with toluidine blue from *A. grandis* microinjected with either *GUS* dsRNA or *AgraCHS2* dsRNA, as indicated. In (D), the arrow points to damaged tissue in the midgut due to *AgraCHS2* RNAi-mediated gene silencing. Scale bars in (A and B) = 2 mm and in (C and D) = 100 μm.

Alignment and phylogenic analysis suggested that *AgraCHS2* belongs to the CHS2 group.

Chitin synthase enzymes are located in the cell plasma membrane and participate in chitin chain translocation in the extracellular space (Broehan et al., 2007; Moussian et al., 2015). Similar to other insects CHSs, AgraCHS2 is predicted to be an integral membrane protein harboring 15 transmembrane segments. The disposition and a conserved number of these membrane-spanning regions in CHS allow the central domain (domain B), which is the catalytic domain, to face the cytoplasm, where the UDP-N-acetyl-glucosamine substrate is accessible (Tellam, Vuocolo, Johnson, Jarmey, & Pearson, 2000). Its catalytic domain has a high degree of conservation among insect CHSs and includes the conserved residues from chitin synthase family, EDR, and QRRRW (Merzendorfer, 2006; Tellam et al., 2000). Among its fifteen predicted transmembrane segments, AgraCHS2 has five segments that are located immediately after the central catalytic domain, forming a topology termed the TMS 3–5 region. This topology is present in both insect and fungal CHSs (Merzendorfer, 2011). Further *in silico* analysis predicted that AgraCHS2 is a large and complex integral membrane protein with an intrinsic potential for multiple post-translational modifications, such as N- and O-glycosylation, which may be involved in the regulation of its activity. In addition to other CHS2 sequences, the AgraCHS2 protein sequence does not present regions with the potential to form coiled-coil structures after the TMS 3–5. Coiled-coil structures are present only in CHS and are related to interactions with proteins that regulate the activity of CHS1 and/or vesicular traffic within cells (Carpita, 2011; Melia et al., 2002).

In this study, a single copy of the *AgraCHS2* gene in the *A. grandis* genome was identified. The presence of a single AgraCHS2 copy is consistent with observations in other insect species (Ampasala et al., 2011; Arakane et al., 2005; Gagou et al., 2002; Hogenkamp et al., 2005; Tellam et al., 2000; Zhu et al., 2002). A single-copy gene is a favorable characteristic for engineered gene silencing, as no other gene can compensate for the lack of CHS in the organism.

The expression pattern of *AgraCHS2* was studied during all *A. grandis* developmental stages by qPCR. A higher expression level of *AgraCHS2* was observed in third-instar larvae, which is the period that insects feed the most (Fig. 6A). Low levels of transcripts were observed in the egg and pupal stages, which are periods of insect starvation. Similar results were observed upon analyzing CHS class 2 expression in *Spodoptera frugiperda* (Lepidoptera: Noctuidae), *Spodoptera exigua* and *T. castaneum*. The *AgraCHS2* gene is mainly expressed in *A. grandis* midgut, suggesting that it plays a key role in the chitin synthesis of the PM in *A. grandis* (Arakane et al., 2008; Bolognesi et al., 2005; Hogenkamp et al., 2005).

To evaluate the effects of RNAi-mediated silencing of the *AgraCHS2* gene in the PM, insect adult females were microinjected with *AgraCHS2* dsRNA. These assays revealed promising results for controlling this insect pest. The RNAi-mediated silencing of *AgraCHS2* had no effect on the larva-to-pupa-to-adult transitions when the microinjection was performed in the last larval instar. However, when the microinjection was performed in adult females, severe effects on mortality and reproduction were observed. Ten days after *AgraCHS2* dsRNA microinjection, total mortality was observed. The persistence of gene silencing for 10 days supports the hypothesis of a mechanism that maintains the RNAi effect (Miller, Miyata, Brown, & Tomoyasu, 2012). It was also observed that those females microinjected with *AgraCHS2* dsRNA laid significantly fewer eggs, and oviposition was no longer observed 10 days' post-microinjection because the insects had died. In general, *AgraCHS2* RNAi-mediated gene silencing resulted in a 93% reduction in oviposition. Similar results were reported for *CHS2* silencing experiments in *T. castaneum* (Arakane et al., 2008). Hence, there is strong evidence that these results reflect an indirect effect of starvation due to lack of a functional PM, which disturbs the process of nutrient assimilation (Kelkenberg et al.,

2015) and thus diverts energetic investment from nonessential functions, such as reproduction (Arakane et al., 2008).

The adult insects microinjected with *AgraCHS2* dsRNA stopped feeding (data not shown). Similar behavior was observed in insects fed WGA lectin, which binds to the PM and causes pore formation in these structures (Harper, Hopkins, & Czapla, 1998; Levy, Falleiros, Moscardi, & Gregorio, 2011). The lack of a functional PM allows contact of the bolus particles with the gut epithelium, causing injury, as observed in the histological images of the *A. grandis* midgut. In response to this mechanical damage in the gut epithelium, the insects stop feeding (Hopkins & Harper, 2001).

Therefore, the *AgraCHS2* gene is essential for *A. grandis* PM biosynthesis. The PM is essential for the protection of the intestinal epithelium and for nutrient assimilation. Hence, malfunction of this structure caused by RNAi-mediated gene silencing resulted in a lethal phenotype (Kelkenberg et al., 2015). The findings presented in this work suggest that *AgraCHS2* is a potential and essential molecular target for RNAi-mediated gene silencing to be used in biotechnological insect pest control strategies, which can include the use in genetic transformation of cotton plants, or by non-transforming methods, such as in the use of formulations for dsRNA delivery (Joga et al., 2016).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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