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Transcriptomic Survey of the Midgut of *Anthonomus grandis* (Coleoptera: Curculionidae)

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ABSTRACT. *Anthonomus grandis* Boheman is a key pest in cotton crops in the New World. Its larval stage develops within the flower bud using it as food and as protection against its predators. This behavior limits the effectiveness of its control using conventional insecticide applications and biocontrol techniques. In spite of its importance, little is known about its genome sequence and, more important, its specific expression in key organs like the midgut. Total mRNA isolated from larval midguts was used for pyrosequencing. Sequence reads were assembled and annotated to generate a unigene data set. In total, 400,000 reads from *A. grandis* midgut with an average length of 237 bp were assembled and combined into 20,915 contigs. The assembled reads fell into 6,621 genes models. BlastX search using the NCBI-NR database showed that 3,006 unigenes had significant matches to known sequences. Gene Ontology (GO) mapping analysis evidenced that *A. grandis* is able to transcripts coding for proteins involved in catalytic processing of macromolecules that allows its adaptation to very different feeding source scenarios. Furthermore, transcripts encoding for proteins involved in detoxification mechanisms such as *p450* genes, *glutathione-S-transferase*, and *carboxylesterases* are also expressed. This is the first report of a transcriptomic study in *A. grandis* and the largest set of sequence data reported for this species. These data are valuable resources to expand the knowledge of this insect group and could be used in the design of new control strategies based in molecular information.

Key Words: 454 pyrosequencing, cotton pest, midgut expressed gene

The cotton boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) was first reported in 1880 in Mexico, and years later in Texas (USA) (Burke et al. 1986; Kyung and Sappington 2004). Dispersion has hypothesized to have occurred from Mexico to the north along the Pacific and Gulf coasts. Unlike its natural dispersion in North America, *A. grandis* may have arrived in South America several times through the cotton trade (Manessi 1997). The first record of this pest in South America was described in 1949 in Venezuela, while in the southernmost country, Argentina, its presence was detected more recently (in 1993; Stadler and Buteler 2007). Nowadays, it is one of the most serious pests that affects cotton-growing region in South America.

Under optimal conditions, the total life cycle of *A. grandis* may last up to 3 weeks and, depending on environmental conditions, 7–10 annual generations may occur. Regarding oviposition, *A. grandis* females tend to prefer squares over bolls as oviposition substrates; each female oviposits up to 300 eggs, which are individually deposited in flower and cotton buds that serve as shelter and food for the larvae (Showler 2007). Although usually associated with cultivated cotton (*Gossypium hirsutum* L.), *A. grandis* has been found to feed and reproduce on alternative hosts including species within the genera *Gossypium, Cienfuegosia, Thespesia, Hibiscus*, and *Hampea* (Burke et al. 1975, Cuadrado and Garralla 2000). These elaborated eating habits in a polyphagous species such as the boll weevil hypothetically lead to the regulated expression of many genes in the midgut that allows the insect to adapt to different sources of food. However, no transcriptomic, proteomic, or metabolomics studies have been previously performed to study its gene repertoire and regulation. Available genetic sequences in the Order Coleoptera are strikingly reduced considering the biological and agronomic importance of the group. Up to date, only the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) and the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae) genomes have been completely sequenced (Richards et al. 2008, Keeling et al. 2013). Transcriptome analyses have described expressed genes and putative proteins in coleopteran as *Callosobruchus maculatus* (Pedra et al. 2003), *T. castaneum* (Morris et al. 2009) and the mountain pine beetle, *D. ponderosae* Hopkins (Keeling et al. 2012), while little genomic data from *A. grandis* is still available. The few available studies on this species have focused on individual genes associated with the development (Taban et al. 2006) or the interaction with toxic proteins from *Bacillus thuringiensis* (Martins et al. 2010). Regarding the evaluation of new tools to control this pest, genes encoding for digestive enzymes like serine proteinases and their inhibitors have been also studied (De Oliveira Neto et al. 2004, Gomes et al. 2005, Martins et al. 2010, Nakasu et al. 2010).

The use of chemical insecticides is currently the main strategy to control *A. grandis* (Showler 2007). In other insect pests controlled by this method, the study of gene products associated with detoxification processes as cytochrome P450 (CYP), glutathione transferase (GST), and carboxylesterases (COEs) has been used to detect the occurrence of resistant populations (Yang et al. 2006, Ramoutar et al. 2009, Siegwart et al. 2011). In coleopteran, a recent study showed that a *p450* gene, predominantly expressed in the brain, is responsible for the majority of deltamethrin resistance in *T. castaneum* (Zhu et al. 2010).

The constant increase of genomic data has expanded not only the knowledge about biological processes, but also the development of new management strategies in pest control. For instance, use of RNA interference (RNAi) to block the expression of essential genes in insect pests (Tomoyasu and Denell 2004, Hrycaj et al. 2008, Minakuchi et al. 2008) is a recent example of a genomics-derived, pest control strategy the RNAi. The administration of double-stranded RNA (dsRNA) by injection or by oral route has proven to be an efficient method for functional studies. RNAi has a high potential as alternative and efficient method of control of insects (Whyard et al. 2009). Previous studies have shown that *V-ATPase A* gene silencing can be induced in the

beetle *Diabrotica virgifera virgifera* by dsRNA applied on diet or produced in transgenic plants (Baum et al. 2007). The data obtained by Baum et al. show the potential use of RNAi technology in crop protection and the importance of genetic information in the design of control strategies.

In this study, a Roche 454-based pyrosequencing method was used to define the larval midgut transcriptome of *A. grandis*, a major agricultural pest in the New World. This is the first highly precise description of the diversity of synthesized mRNAs along with their putative functionality deduced in silico. The 400,000 reads assembled in 20,915 contigs represent an important improvement in terms of genomic information for this species. In fact, up to date, only 215 *A. grandis* mRNA sequences are reported in Genbank.

Materials and Methods

***Anthonomus* Rearing and Midgut RNA Isolation.** *A. grandis* larvae were reared at the Institute of Microbiology and Agricultural Zoology (IMYZA), INTA. Larvae were raised on a cotton-based artificial diet at 28°C, 70 % relative humidity, with a 12-h light/darkness photoperiod (Lecuona 2009). For RNA extractions, sets of 15 midguts were dissected and washed to eliminate diet residues present in midgut lumen from *A. grandis* larvae, and total RNA was isolated using TRIZOL reagent kit (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was stored in 20 µl double distilled diethylpyrocarbonate (DEPC) treated water and the concentration was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) following the manufacturer's guidelines.

Sequence Trimming, Assembly of ESTs, and Annotation. Reads were generated by pyrosequencing in a 454 automatic sequencer using the services offered by Life Sequencing S.L. (Parc Científic Universitat de València, Spain). The sequences were subjected to filtration to remove duplicates (reads that begin exactly at the same position), and to exclude the sequences that failed to fit the following criteria: minimum average read quality >20, minimum read length >50, and minimum read tail quality >18. The assembly step was performed using MIRA3 assembler (Chevreux et al. 2004). High-quality sequences were assembled to 20,915 contiguous sequences (contigs) consisting of two or more sequences of *A. grandis.* Then, two different ORF predictors were run over the contigs: GLIMMER (Delcher et al. 1999), with a self-trained protein model, and AUGUSTUS (Stanke et al. 2004) with *T. castaneum* protein model. The outputs of both predicted programs were considered to build the initial Orfeome Catalogue for *A. grandis*. Predicted genes models annotation and GO term mapping were done using Blast2GO. BlastX searches to assign a putative function were run against NCBI-NR (National Center for Biotechnology Information, NCBI) with an e-value cut-off of 1^{e-6} (Altschul et al. 1990).

Phylogenetic Analysis. A phylogenetic tree was constructed based on the nucleotide sequences of cathepsin genes (up to 300 nt) obtained from *A. grandis* midgut. Sequences were independently aligned using ClustalX program (Thompson et al. 1997). After completing individual alignments, phylogeny was inferred using MEGA 5 program (Tamura et al. 2011) with the following parameters: Neighbor-Joining (NJ) method; Bootstrap with 1,000 replicates. A phylogenetic tree was constructed based on the amino acid sequences of cathepsin from *A. grandis* (contig 230, accession number JR948171.1) and different insect orders. Sequences were independently aligned using ClustalX program, with the following parameters: Pairwise alignment (Gap Open Penalty = 10, Gap Extension Penalty = 0.1, protein weight matrix: Blosum 30); Multiple alignment (Gap Open Penalty = 10, Gap Extension Penalty = 0.05, protein weight matrix: Blosum series). Then complete individual alignments and phylogeny was inferred using MEGA 5 program with the following parameters: NJ method; Bootstrap with 1,000 replicates; gap/Missing data = complete deletion; Model = Amino (Dayhoff Matrix); patterns among sites = Same (Homogeneous); rates among sites = Different (Gamma Distributed); gamma parameter = 2.25.

Microsatellites Identification and Analysis. Microsatellites identification and analysis were performed using SciRoKo 3.4 software (Kofler et al. 2007) with the default parameters. Sequence inputs were delivered in FASTA format. Sequences that included microsatellites were analyzed using Blast2GO in order to know the putative functions associated.

Sequence Submission. The raw data obtained were submitted to the Short Read Archive database at NCBI with accession number SRX116057. The complete data set obtained by 454 sequencing were submitted to the NCBI Genbank with accession number JR948021–JR950755.

Results

Assembly of Isotigs and Annotation of Midgut Transcriptome. As described in materials and methods, total RNA was extracted from *A. grandis* larvae midguts. In total, 400,000 reads with an average length of 237 bp were obtained using GS FLX Titanium 454 technology (Roche), which is based on a pyrosequencing method. Reads were assembled into 20,915 contigs using Mira3 assembler under default parameters. After ORF prediction using Glimmer and Augustus programs, the assembled sequences fell into 6,621 unique gene models that were annotated using Blast2Go program (Conesa et al. 2005; Table 1). BlastX search was performed using the NCBI-NR protein database with a cut-off E-value of 10^{-6}; the results showed that 3,006 gene models have significant matches to already characterized heterologous sequences. In addition, *D. ponderosae* (Coleoptera: Curculionidae) was the species with the highest number of identities (Supp Fig. S1). The remaining mRNA sequences translated in silico exhibited no significant match against protein databases, suggesting that many *A. grandis* gut transcripts represent novel coleopteran sequences.

In total, 3,006 unigenes were assigned into the GO categories (biological process, cellular component, and molecular function) using Blast2GO. Biological process made up the majority of the GO annotations with 4,404 GO term assigned, followed by cellular component (2,799 terms) and molecular function (2,531 terms; Fig. 1). The molecular function category (Fig. 1A) was mainly comprised of catalytic activity (36.6 %) and binding activity (37.32 %) proteins as expected for the midgut tissue. Structural molecules (13.86 %) and transporters (6.19 %) accounted for an important fraction of the total. In addition, minor groups included electron carriers and proteins with antioxidant activity. Among biological process, metabolic (26.78 %) and cellular processes (24.10 %) were the most dominant subcategories (Fig. 1B). Under the category of cellular component, cell (35.80 %), organelle (27.15 %) and macromolecular complex component (24.46 %) were among the most highly represented subcategories (Fig. 1C).

Genes Putatively Related to Larval Midgut Physiology. The insect's gut plays essential roles in food digestion, defense against pathogens and biological insecticides, detoxification of chemical, and enzyme inhibitors from plants. By means of sequence analysis obtained from *A. grandis* midgut, a wide variety of putative proteins involved in these processes have been found. These genes are main targets as candidates for biotechnological control strategies based on PTGS/RNAi induction. With the help of these technologies, the effect of the downregulation of

Table 1. Summary statistics for *Anthonomus grandis* midgut expressed sequence tag assembly

Total number of reads	3,41,485
Total number of reads in the assembly	1,45,893
Total number of contigs	20,915
Total number of singletons	1,95,592
Average read length	327 pb
Number pre-edited genes	7,500

these proteins on host survival can be assessed. Midgut receptors are also well known to be relevant in the insecticide effectiveness of *B. thuringiensis* (*Bt*) toxins. This work focused on proteins involved in digestion, defense and detoxification, *Bt* toxin binding, and RNAi processes (Table 2).

Digestive Proteins. Transcripts encoding the cathepsin-L group were the most highly represented in *A. grandis* midgut. Cathepsins are cysteine proteases involved in protein catabolism activated at a low pH. In insects, these proteins have been widely described in the alimentary tract but they also play an important role in embryo vitelline metabolism and in the metamorphic process (Takahashi et al. 1993, Cho et al. 1999). Due to its central adaptive role and its relatively good characterization in insects, this gene family was selected to show the diversity found in *A. grandis* midgut larvae. In this study, five Cathepsin classes

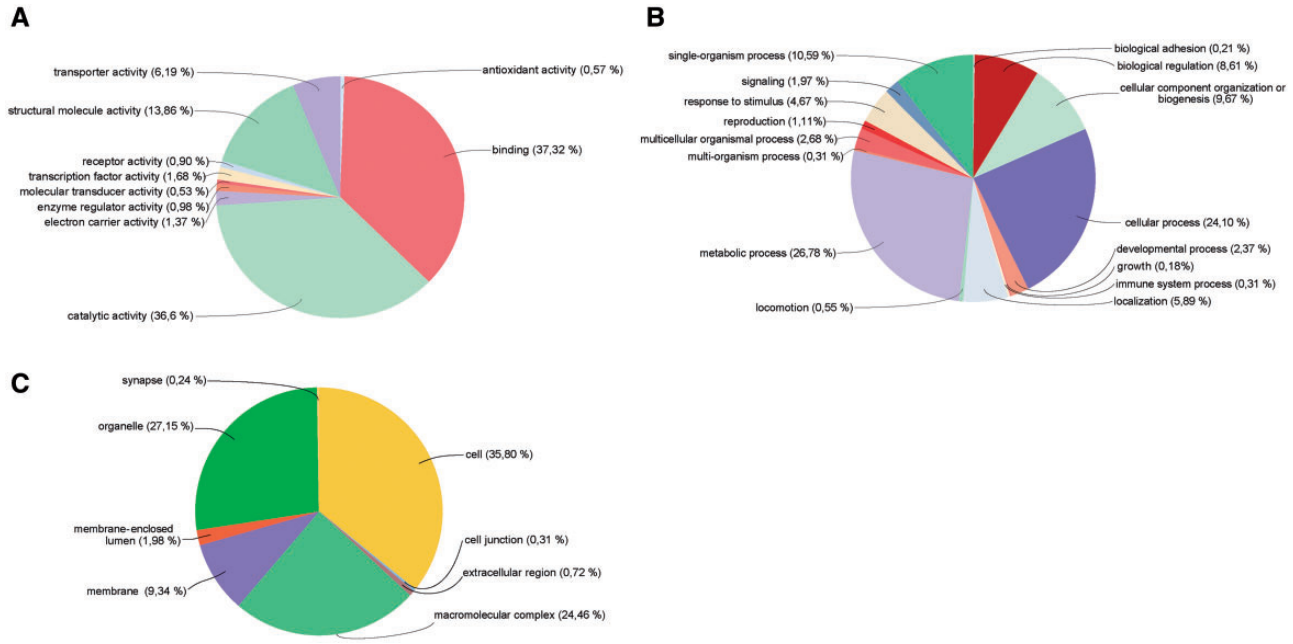

Fig. 1. Gene ontology (GO) assignments for *A. grandis* larval midgut transcriptome. GO terms distribution by (A) molecular functions at level 2, (B) biological processes at level 2 and (C) cellular component at level 2. The number shows the percentage of GO terms included in each. One sequence could be associated with more than one GO term at the same time.

Table 2. Midgut genes related to important physiological functions

	Number of contigs	Min. E-Value	Best mean similarity (%)	Go terms
Digestion				
α-amylase	4	2.05 e−15	85.2	P: carbohydrate metabolic process; P: digestion;
α-glucosidase (maltase)	7	4.87 e−8	73.9	P: carbohydrate metabolic process; F: hydrolase activity
β-glucosidase	6	1.77 e−49	67.7	P: metabolic process; F: hydrolase activity
Carboxypeptidase	8	3.51 e−7	84.5	P: proteolysis; F: metallocarboxypeptidase activity;
Cathepsin all types	98	7.19 e−11	76.6	F: protein serine/threonine kinase activity; P: protein amino acid phosphorylation
Celluloses all types	13	1.99 e−18	81.7	P: carbohydrate metabolic process; F: cellulose catalytic activity
Lipase all types	11	1.31 e−17	69.9	F: hydrolase activity
Serine proteinase all types	12	7.42 e−21	75.3	F: catalytic activity; P: metabolic process
Detoxification and defense				
Carboxylesterase	11	5.87 e−10	79.5	F: hydrolase activity
Cytochrome p450	23	5.12 e−6	71.8	P: oxidation reduction; F: monooxygenase activity
Glutathione s transferase e3	15	6.61 e−38	63.0	F: glutathione transferase activity
Superoxide dismutase	2	9.90 e−10	61.5	P: cellular macromolecule metabolic process; F: superoxide dismutase activity
Lysozyme	4	9.68 e−34	66.6	F: lysozyme activity; P: antimicrobial humoral response
Serpin	3	8.80 e−19	73.0	P: negative regulation of endopeptidase activity
Transferrin	1	9.36 e−24	72.6	P: cellular iron ion homeostasis
Peptidoglycan recognition protein	1	2.79 e−66	74.5	P: defense response to Gram-positive bacterium
Bt toxins-binding proteins				
Cadherin-like	1	3.45 e−10	56.30	
Aminopeptidase N	4	5.17 e−11	67.1	F: peptidase activity, acting on L-amino acid peptides
RNA interference				
Argonaute	2	4.63 e−58	68.3	F: nucleic acid binding; F: translation initiation factor activity
Sid	2	1.98 e−35	57.9	F: hydrolase activity

To analyze the wide range of putative proteins found in the midgut of larvae of *A. grandis* this study focused on four groups of gene that encode functions relating to digestion, defense and detoxification, Bt toxin binding and RNA interference processes. F: function; P: process.

(L, A, K, D, and B) were described and their interrelationship was also analyzed (Fig. 2). By means of alignments with proteins homologous to this protein, a C1A domain was found between the amino acids 327 and 365 of a precursor cathepsin L found in *A. grandis.* Conserved catalytic sites can be observed in positions C (351), H (494), and N (514) demonstrating the presence of the typical site domains of this group of proteins (Fig. 3A). A phylogenetic analysis was performed using *A. grandis* cathepsin-L (contig 230 in Fig.2, accession number JR948171.1) with its homologous described in *T. castaneum* as well as in non-coleopteran insects like *Nasonia vitripennis*, *Drosophila melanogaster*, and *Manduca sexta.* The protein sequences grouped in relation with the taxonomic classification; the coleopteran cluster joined to sequences from Hemiptera, Dictyoptera, Phthiraptera, and Hymenoptera (Fig. 3B).

The study of catalytic proteins present in the digestive tract of insects have allowed the development of pest control strategies based on the use of inhibitors (De Oliveira Neto et al. 2004; Gomes et al. 2005), RNA silencing (Zhou et al. 2008), or transgenic plants (Pitino et al. 2011).

Herbivorous species as the cotton boll weevil have a broad diversity of enzymes involved in sugar metabolism such as glycolysis and gluconeogenesis, nucleotide sugar metabolism, and cellulose degradation. In order to indentify different metabolic pathways present in the midgut of *A. grandis,* deduced proteins with a high relative abundance were analyzed and annotated using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) terms (Kanehisa and Goto 2000) (Fig. 4A–C). These data together with homologous sequences obtained from

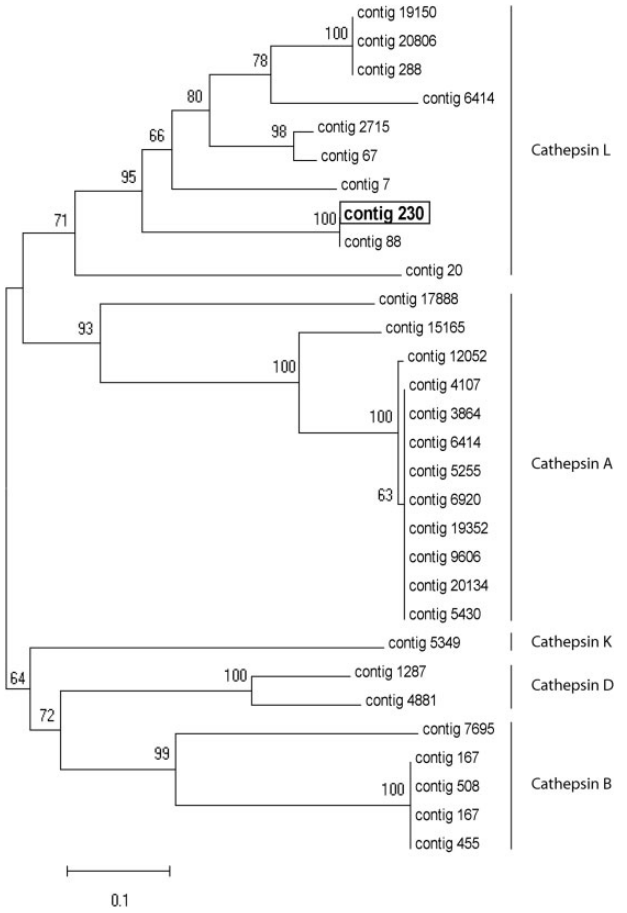

Fig. 2. Diversity and relationships of A. grandis cathepsins. Homologous nucleotide sequences (>300 pb) to Cathepsins obtained from *A. grandis* midgut were aligned and used to analyze a phylogenetic tree in order to know the diversity within each class and between classes.

T. castaneum database were compared and analyzed. The presence/absence of enzymes included in different metabolic pathways such as glycolysis and gluconeogenesis are presented in order to compare similar or alternative routes used in coleopteran (Supp Fig. S2).

Many enzymes were represented including amylases, hexokinases, cellulases, among others. Importantly, celluloses possess potential uses in industrial applications (Kuhad et al. 2011). Putative cellulases, e.g. were represented with members of different enzyme groups as beta-cellobiosidase, beta-glucosidase, and beta-endoglucanase. Regarding account similar cellulases described in other coleopteran species, identity values ranged from 33% to 85 %. Research to evaluate the activity of these enzymes is in progress.

Putative Defense and Detoxification Proteins. The digestive tract of insects represents the entry for food but also of pathogenic microorganisms such as bacteria, fungi, and viruses. Chemical substances such as insecticides or plant toxins also enter through this way. Processes of detoxification to xenobiotics and endogenous compounds are mediated by different groups of detoxifying enzymes (Dowd and Spark 1983, Yu 2005). Most studies related to the elimination of chemical pesticides focused on three enzyme families: CYPs, GST, and COEs that are responsible for metabolic resistance in insects. At the moment, cotton boll weevil is mainly controlled by chemical insecticides; which has led to the emergence of resistance in field population (Graves et al. 1967). In this study, we describe representative putative proteins that could be analyzed to detect the resistance emergence:

The CYP superfamily includes many genes clustered in different families represented in all organisms. To date, insect P450s have been assigned to six CYP families: five are insect specific (CYP6, 9, 12, 18, and 28) and one, CYP4, includes sequences from vertebrates (Feyereisen 1999). Most insect CYP proteins are involved in different processes such as oxidation of organic substances, metabolic intermediation of lipids, and the metabolism of xenobiotic substances of natural or synthetic origin (Stevens et al. 2000). A recent study showed that CYP overexpression increased the metabolism of insecticides leading to resistance in the insect (Yang et al. 2006). Furthermore, a *p450* gene have been identified and characterized as responsible for the deltamethrin resistance in *T. castaneum* (Zhu et al. 2010). Recent reports showed that larvae fed with plant material expressing dsRNA silencing *p450* gene increased the susceptibility to gossypol, a defence substance from plants in the insect (Mao et al. 2007, Bautista et al. 2009).

In this study, we found that 23 contigs have significant similarity with P450s. The families represented in this analysis were CYP4, CYP6, and CYP9. CYP9 showed larger relative abundance (11 contigs). Alignments using homologous proteins from coleopteran showed sequence variations within functional important domain (Supp Fig. S3). Previous studies have shown that CYP9 has a role in the metabolism of foreign compounds including chemical insecticides (Rose et al. 1997, Stevens et al. 2000). In this context, it was reported that CYP genes are upregulated in presence of xenobiotic or insecticidal substances through both constitutive overexpression and induction mechanisms (Poupardin et al. 2010, Liu et al. 2011).

Glutathione-S-transferase (GST): GSTs play a central role in the detoxification of both endogenous and xenobiotic compounds and they are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress (Gullipalli et al. 2010, Huang et al. 2011). In insects, there are two major classes of GSTs classified according to their location within the cell: microsomal and cytosolic. Most studies have been focused on cytosolic GSTs and the highly diverse insect genes have been divided into six major subclasses sharing at least 60% aminoacidic identity: Delta, Epsilon, Sigma, Omega, Theta, and Zeta. Delta and Epsilon classes are insect-specific, while the other subclasses have a broad taxonomic distribution (Ranson and Hemingway 2005, Low et al. 2007). The role of GSTs in the resistance to chemical insecticides was well established in Coleoptera (Kostaropoulos et al. 2001, Ramoutar et al. 2009). The analysis of *A. grandis* transcriptome indicates that 15 contigs have significant blast

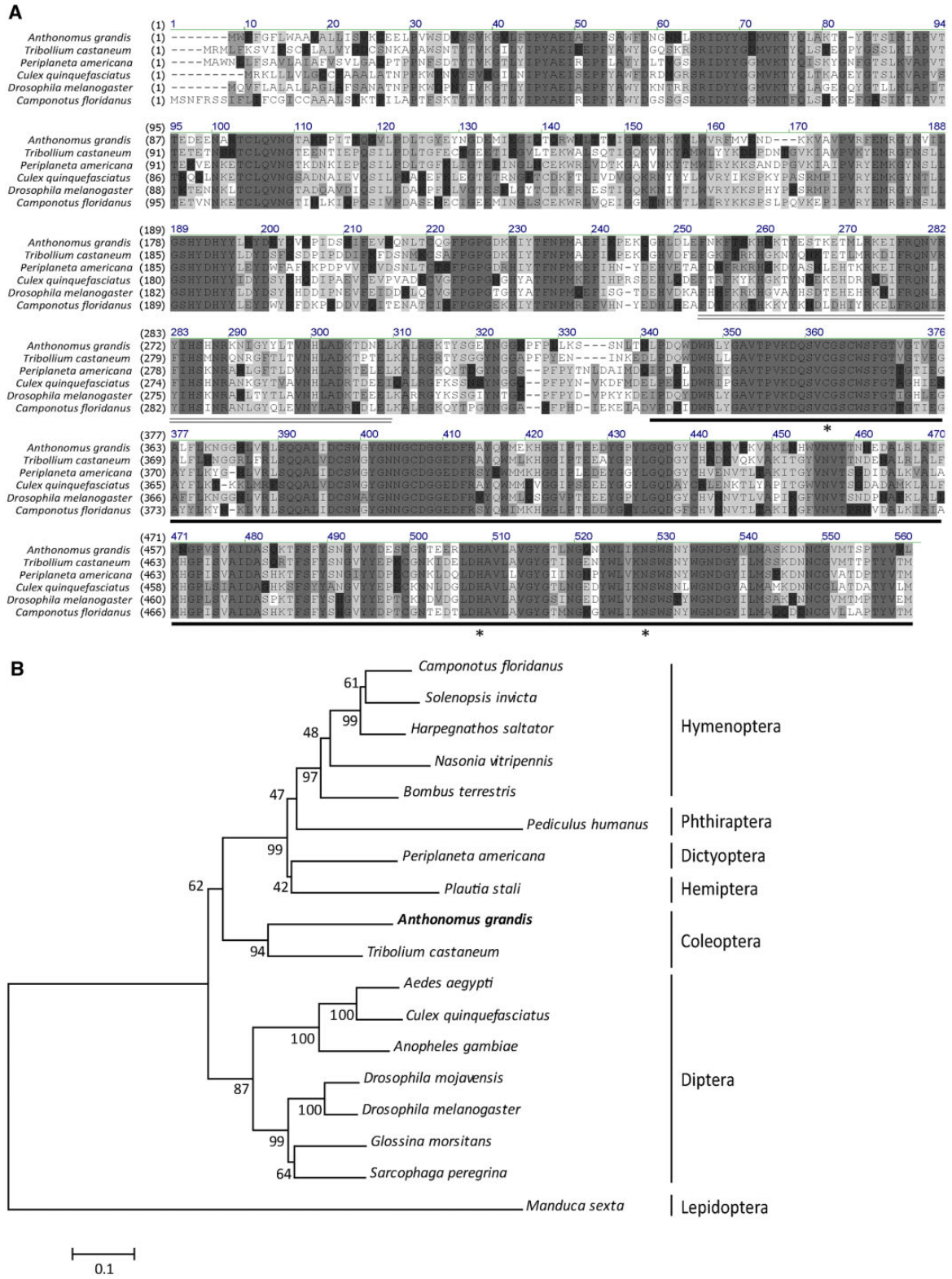

Fig. 3. Multiple alignments of *A. grandis* cathepsins and dendrogram. Cathepsin-L from *A. grandis* (Contig 230, accession number JR948171.1) and representatives organisms were individually aligned. Phylogenetic tree was inferred with MEGA 5 program. (A) The C1A domain is underlined and the catalytic sites are marked with an asterisk. Propeptide inhibitor domain is double underlined. Identical residues are boxed with dark shading. (B) Phylogenetic tree showing relationships between Cathepsins L. from organisms of different orders included in Insecta Class: *Tribolium castaneum* (NP_001164088.1), *N. vitripennis* (XP_001605879.1), *Bombus terrestris* (XP_003402785.1), *Periplaneta americana* (BAA86911.1), *Camponotus floridanus* (EFN68284.1), *Drosophila melanogaster* (NP_620470.1), *Drosophila mojavensis* (XP_002008774.1), *Anopheles gambiae* str. PEST (XP_307325.4), *Glossina morsitans morsitans* (ABC48937.1), *Sarcophaga peregrina* (BAA76272.1), *Solenopsis invicta* (EFZ13575.1), *Aedesaegypti* (XP_001657758.1), *Culex quinquefasciatus* (XP_001867470.1), *Harpegnathos saltator* (EFN82144.1), *Plautia stali* (BAF94153.1), *Pediculus humanus corporis* (XP_002425065.1), and *Manduca sexta* (CAX16636.1). Node support is indicated by bootstrap values.

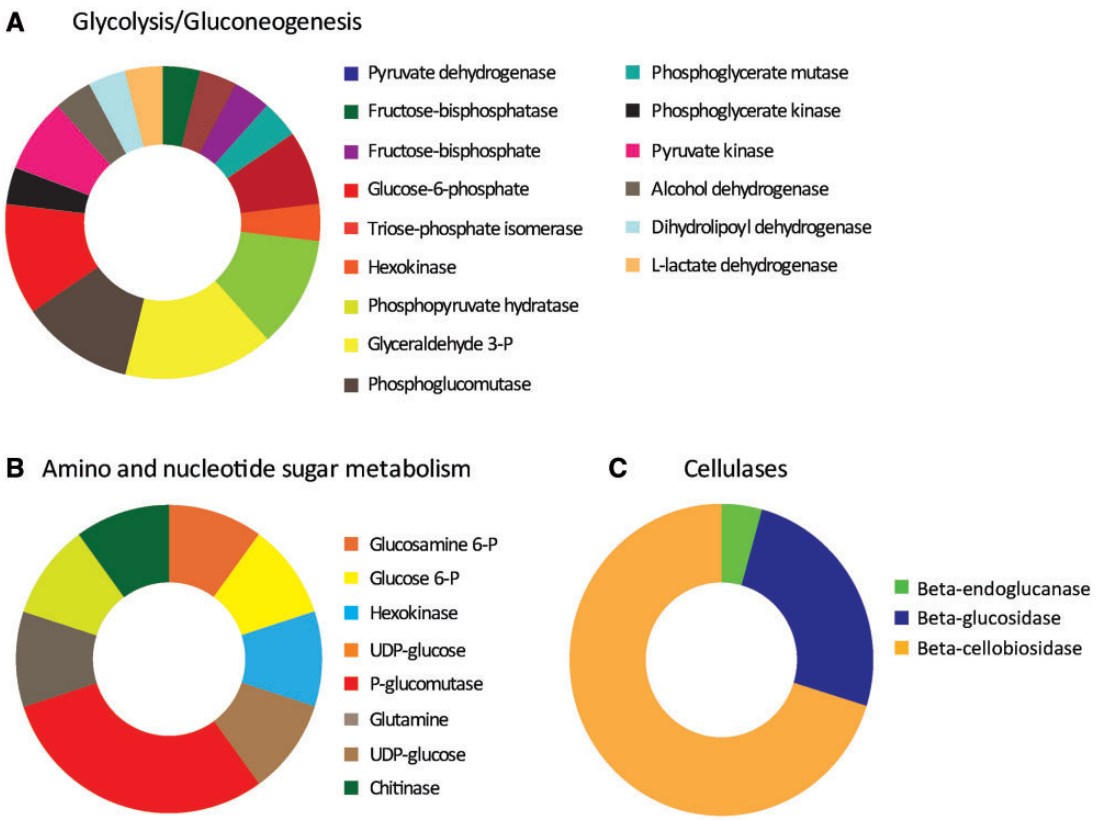

Fig. 4. Carbohydrate metabolism. Major metabolic pathways associated with sugars were analyzed indicating the enzymes found in the *A. grandis* intestine and their relative abundance. (A) and (B) were used as reference the Keggs terms of each metabolic pathway. (C) variety of cellulases found and their relative abundances.

with GTSs from different insects. The Sigma Class was the relatively most abundant, followed by Delta–Epsilon classes. The Sigma Class is associated with different oxidative stress processes, while Delta–Epsilon classes are involved in insecticide detoxification processes (Ortelli et al. 2003, Lumjuan et al. 2005).

Cellular carboxylesterases (COEs): These proteins hydrolyze esters of carboxylic acids. Thus, they have a broad range of functions in catabolism of pheromones, juvenile hormone and in the hydrolysis of the neurotransmitter acetylcholine (Riddiford et al. 2003, Oakeshott et al. 2005). However, their participation in the process of resistance to chemical insecticides like organophosphates and pyrethroids has been its most studied role (Hemingway and Karunaratne 1998, Yu et al. 2009). The study of COE gene sequences allowed an increased knowledge of insecticide resistance mechanisms which were associated with site-specific mutations. Such mutations resulted in decreased insecticide activity or a higher insecticide metabolism by overexpression of the enzymes (Cui et al. 2007, Li et al. 2007). Previous studies have shown that strains of *T. castaneum* susceptible to the insecticide Malathion have an increased activity of COE associated with a higher affinity of the enzyme to the substrate (Haubruge et al. 2002). The COE sequences found in *A. grandis* showed an amino acidic identity ranging between 63% (JR948289.1) and 86% (JR950524.1) with *D. ponderosae*. The low identity found in some COE sequences by blast could be indicates the presence of novel enzymes to be included in this protein family. To date, there are no studies about the role of detoxification proteins mentioned above play in the insecticides metabolism in *A. grandis*.

***Bt* Toxin-Binding Proteins.** *B. thuringiensis* crystal toxins (*Bt*) are the insecticide biomolecules most extensively used to control insect pests. Most *Bt* toxins are active to insects from the Order Lepidoptera but Cry3 and Cry8 classes are active against Coleoptera (Bravo et al. 1998). *Bt* toxins act at the midgut membrane level after previous activation by proteases present in the intestinal juice. The active protein binds to receptors on the membrane of intestinal cells where they cause the formation of pores that lead to osmotic imbalance and ultimately to the death of the insect (Rajamohan et al. 1998). Two proteins were found to be involved in binding and subsequent processing of the toxin: cadherin-like proteins and N-aminopeptidases (Rajagopal et al. 2002, Flannagan et al. 2005, Yang et al. 2010). Both classes of proteins are fully represented in the *A. grandis* isotig data set (Table 2). The only cadherin sequence identified here showed best blast with a homologous sequence from *D. ponderosae* with an identity of 69%. This low number of cadherin variants may explain the relative failure to screen for effective *Bt* toxins for the cotton boll weevil as compared to other insect species. There is only one successful report of a *Cry* gene encoded *Bt* protein able to affect *A. grandis* (Grossi de Sa et al. 2007, Martins et al. 2008). Moreover, the receptors that could be involved in *Bt* binding activity have also been reported (Martins et al. 2010, Nakasu et al. 2010). The rather few receptor variants makes this protein an interesting target for their study. The comprehension of the molecular basis of toxin-receptor binding could be useful to develop recombinant Cry toxins with different specificities and thus improve the toxic activity.

Putative Proteins Associated With RNAi Regulation Mechanism. In *T. castaneum*, the injection of dsRNA into late instar larvae produced a systemic RNAi effect from the injection site to other tissues (Tomoyasu and Denell 2004, Tomoyasu et al. 2008). While similar results were obtained with *C. elegans* (Fire et al. 1998), these results were not observed in *Drosophila sp* (Winston et al. 2002, Roignant et al. 2003). Putatively encoded proteins similar to SID-1 proteins, which are involved in the dsRNA uptake in *C. elegans* (Winston et al. 2002) are present in the transcriptome of *A. grandis*. Putative SID-1 protein encoding sequences showed the best blast with homologous sequences of *T. castaneum*. It is interesting to note that argonaute proteins that

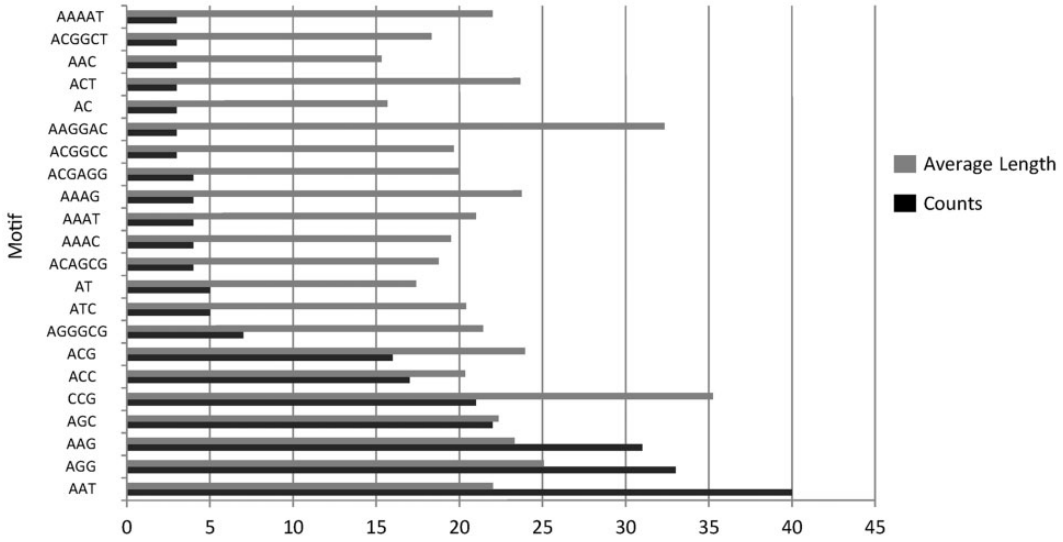

Fig. 5. Microsatellite. Distribution of most abundant microsatellite motifs indicating the number of scorings.

have a central role in gene silencing pathways (Hock and Meister 2008) are also present in *A. grandis*. We found five contigs with significant identity to argonaute proteins. These findings indicate that is possible to evaluate strategies based in gene silencing applied to functional studies or control pest. This control strategy is being evaluated in larvae and adults of *A. grandis* (unpublished data).

Identification of Microsatellites (SSRs). The use of molecular markers is now a current practice for population genetic studies like epidemiologic characterization. More recently, functional markers (as opposed to the most common neutral markers) have become useful tools to study evolutionary processes like selection of resistant populations as a result of insecticide applications.

Transcriptome analysis is not only an efficient approach for gene discovery but also an effective approach for the identification of functional DNA markers, because they are located within the actual genes having strict linkage-disequilibrium with a given trait encoding gene.

They can be found within intergenic regions, protein-coding genes and their untranslated regions (UTRs), or within introns (You-Chun Li et al. 2004). Their presence is associated with effects on the organism's phenotype through the regulation of gene expression and also are used in population studies (Li et al. 2002, Kim and Sappington 2006, Aketarawong et al. 2011).

As explained above, *A. grandis* has most probably originated in Mexico and then it would have been dispersed from there to the rest of the New World. However, there are few molecular markers to study the evolution and the epidemiology of this pest due to the lack of genomic information. Here we identified in silico a total of 272 microsatellites loci with di-, tri-, tetra-, penta-, and hexanucleotide repeats present in different isotigs by using SciRoKo3.4 software. Among these SSRs, the trinucleotide repeats are predominant (76.6%), with (AAT) being the most frequent motif (32.4%; Fig. 5). The microsatellites identified in this study should be validated to elucidate the utility of their analysis.

Analysis performed with Blast2GO restricted to those isotigs where microsatellites were found showed that according to molecular functions 50% of the SSR marked proteins are associated with proteolysis and 50% with binding activity. According to the biological process in which they participate, 19% of SSR containing mRNAs encode for proteins putatively involved in primary metabolic processes and 19% in macromolecular metabolic processes (Supp Fig. S4).

Discussion

A. grandis is widely distributed along of American continent and represents an important pest on cotton production. Control strategies based on chemical methods are difficult due to the emergence of resistance and the endophytic behavior of the larvae (Graves et al. 1967, Showler 2007). Despite its recognized importance, genomic data are still scarce. Therefore, there is a limitation in better understanding the biology of this pest and in developing alternative methods for its control. This study contributes to increase the available genetic information useful for diverse applications.

The wide diversity of mRNAs synthesized in the midgut of *A. grandis* was associated with digestive processes. These putative proteins could serve as targets for specific inhibitors or gene silencing using biotechnological strategies. For instance, sequences identified in this work encoding for putative proteins like carboxylesterases, glutathione transferase, and CYP make feasible the measurement of their levels of expression in field populations. Therefore, they provide valuable tools for early detection of insecticide resistance. With the information generated in this work, it is now feasible to design artificial feeding experiments to study the cotton boll weevil molecular regulation in response to different insecticide applications.

The search for new control strategies based on transgenic plants expressing *Bt* genes requires the knowledge of the structure and molecular diversity of target proteins that may be involved in the processing mechanism and toxin receptor binding. This study sheds new light on the sequence of the putative midgut receptors that may be present in *A. grandis* and its role in susceptibility to the widely used *Bt* toxins.

The putative proteins associated with RNAi mechanisms described here support the feasibility of implementing control strategies against the cotton boll weevil based on gene interference. The synthesis of dsRNAs using selected sequences obtained in this work could be useful to implement new strategies for cotton protection against the cotton boll weevil by transgenic plant dsRNA expression among others.

In addition, the described *A. grandis* microsatellites constitute the largest set of functional markers reported for this species. However, they should be validated and will certainly help to characterize its population dynamics including aspects like epidemiological dispersion patterns.

Finally, we identified sequences encoding enzymes of industrial interest such as cellulases, which are being widely studied in many organisms for their applications in the conversion of cellulose to ethanol.

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