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Pectinases From *Sphenophorus levis* Vaurie, 1978 (Coleoptera: Curculionidae): Putative Accessory Digestive Enzymes

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ABSTRACT. The cell wall in plants offers protection against invading organisms and is mainly composed of the polysaccharides pectin, cellulose, and hemicellulose, which can be degraded by plant cell wall degrading enzymes (PCWDEs). Such enzymes are often synthesized by free living microorganisms or endosymbionts that live in the gut of some animals, including certain phytophagous insects. Thus, the ability of an insect to degrade the cell wall was once thought to be related to endosymbiont enzyme activity. However, recent studies have revealed that some phytophagous insects are able to synthesize their own PCWDEs by endogenous genes, although questions regarding the origin of these genes remain unclear. This study describes two pectinases from the sugarcane weevil, *Sphenophorus levis* Vaurie, 1978 (SI-pectinases), which is considered one of the most serious agricultural pests in Brazil. Two cDNA sequences identified in a cDNA library of the insect larvae coding for a pectin methyltransferase (PME) and an endo-polygalacturonase (endo-PG)—denominated SI-PME and SI-endoPG, respectively—were isolated and characterized. The quantitative real-time reverse transcriptase polymerase chain reaction expression profile for both SI-pectinases showed mRNA production mainly in the insect feeding stages and exclusively in midgut tissue of the larvae. This analysis, together with Western blotting data, suggests that SI-pectinases have a digestive role. Phylogenetic analyses indicate that SI-PME and SI-endoPG sequences are closely related to bacteria and fungi, respectively. Moreover, the partial genomic sequences of the pectinases were amplified from insect fat body DNA, which was certified to be free of endosymbiotic DNA. The analysis of genomic sequences revealed the existence of two small introns with 53 and 166 bp in SI-endoPG, which is similar to the common pattern in fungal introns. In contrast, no intron was identified in the SI-PME genomic sequence, as generally observed in bacteria. These data support the theory of horizontal gene transfer proposed for the origin of insect pectinases, reinforcing the acquisition of PME genes from bacteria and endo-PG genes from fungi.

Key Words: plant cell wall degrading enzyme, insect pectinase, pectin methyltransferase, endo-polygalacturonase, horizontal gene transfer

The cell wall in plants offers structural support to the cell and protection against pathogens and phytophagous organisms. Pectic substances are the major component of the middle lamella and are composed of partially methyl-esterified galacturonic acid residues linked by α -1,4 glycosidic bonds (Kashyap et al. 2001). These substances are naturally degraded by pectinases. Pectin methyltransferase (PME) is responsible for the removal of methyl-ester groups, and endo-polygalacturonase (endo-PG) is responsible for the random hydrolysis reaction of α -1,4 bonds (Reignault et al. 1994, Crelier et al. 2001).

Pectinases and other plant cell wall degrading enzymes (PCWDEs) have been extensively studied in plants, bacteria, and fungi. These enzymes constitute an arsenal that can determine the virulence of pathogens (Rogers et al. 2000). A wide range of microorganisms that produce PCWDEs live in symbiotic relationships in the gut of certain insect species, supplementing the nutritional capacity of the host (Calderon-Cortes et al. 2012). Thus, until a few years ago, all PCWDEs found in insect sources were believed to have an endosymbiotic origin. However, studies have shown that some invertebrates, including insects, can synthesize these enzymes by endogenous genes (Watanabe et al. 1998; Girard and Jouanin 1999; Watanabe and Tokuda 2001, 2010; Allen and Mertens 2008; Celorio-Mancera Mde et al. 2009; Willis et al. 2011).

The first insect pectinases described were a PME and an endo-PG, initially purified from extracts of entire adult specimens of the rice weevil (*Sitophilus oryzae*) (Shen et al. 1996, 1999). Since this discovery, pectinolytic enzymes from phytophagous beetles have been identified in several representative members of the superfamilies Chrysomeloidea

and Curculionoidea (Pauchet et al. 2010, 2014). Pectinases and other PCWDE genes have been identified in several herbivorous insect species with distinct feeding habits, which suggests the involvement of PCWDEs in the evolution of plant–insect interactions (Calderon-Cortes et al. 2012).

In Brazil, the sugarcane weevil (*Sphenophorus levis*) is one of the most economically important agricultural pests from the family Curculionidae. The larvae of this beetle feed on the sugarcane rhizome and stem base, building galleries that eventually cause the death of the plant, which leads to a reduction in productivity of one of the most important agricultural crops in the country (Cerdeira et al. 1999). Unfortunately, there are no effective methods for controlling this coleopteran. Thus, our group has built and sequenced a cDNA library from *S. levis* larvae (F.H.S, unpublished data) to identify new molecular strategies for the biotechnological control of this insect. Sequence analyses have revealed a single full-length PME (GenBank: KF697077) and an endo-PG (GenBank: KF697078) denominated SI-PME and SI-endoPG, respectively. The genomic coding sequences of these enzymes were characterized, and gene expression analysis by real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed in different developmental stages as well in different larval tissues. Phylogenetic analyses were also performed to investigate the evolutionary relationships of both gene families.

Materials and Methods

Clone Isolation and Characterization. Clones were obtained from an *S. levis* cDNA library constructed from an RNA of a pool of larvae

reaching the pupal stage (~30-d-old larvae) using the CloneMiner kit (Invitrogen, CA) and 5'-sequenced using DYEnamic ET Dye Terminator Kit in a MegaBACE 1000 Automatic Sequencer (GE Healthcare, USA). After data processing and the assembly of clusters in the dCAS platform (Guo et al. 2009), the pectinase clones were identified using BLASTX and tBLASTX (<http://www.ncbi.nlm.nih.gov/blast>). The clones were sequenced entirely and the amino acid-deduced sequences were analyzed in the SIGNALP 4.0 (Petersen et al. 2011), NetOGlyc 3.1 (R. Gupta, E. Jung, and S. Brunak, unpublished data), and NetOGlyc 1.0 programs (Julenius et al. 2005).

Multiple Sequence Alignment and Phylogenetic Analyses. Multiple alignment was carried out using homologous sequences selected from the NCBI-GenBank database with the aid of the Multalin program (Corpet 1988) with default settings. The sequences were selected to investigate the evolutionary trends of PME and endo-PGs using organisms from distinct taxa. Analyses were performed using 36 PME sequences and 34 endoPG sequences.

To infer evolutionary relationships, multiple alignments were carried out in the MUSCLE program, version 3.8.31 (Edgar 2004a,b), using default parameters and the same dataset. Phylogenetic analyses were performed in MEGA 5.0 (Tamura et al. 2011) using the neighbor joining method (Saitou and Nei 1987) and the Poisson correction model. Regions with gaps and missing data were excluded from the analysis. The robustness of the tree was assessed by 1,000 bootstrap pseudoreplicates. The final graphic representation of the phylogenetic tree (Figs. 2 and 3) was created in Adobe Illustrator v. 6.0.

Analysis of Relative Gene Expression of SI-Pectinases. The analysis of the transcript levels of the SI-pectinases genes was performed as described by Fonseca et al. (2012). Seven development stages of *S. levis* and six different tissues from 30-d-old larvae were analyzed. RNA was extracted from eggs, larvae 10 d after eggs hatching, 20-d-old larvae, 30-d-old larvae, prepupae, pupae, and female adult insects using the Trizol reagent (Invitrogen, CA). RNA integrity was verified in a 1.5% agarose gel, and quantification was performed in a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., DE). The analysis of the expression of pectinases in specific tissues was conducted using RNA extracted from a pool of four 30-d-old larvae, previously externally washed three times in PBS buffer, pH 7.0, immobilized on ice for 5 min and dissected into hemolymph, fat body, head, midgut, hindgut, and integument. RNA was treated with amplification-grade RNase-Free DNase I (Invitrogen, CA) and a representative pool of 600 ng containing equal amounts of RNA from three different specimens in the same development stage and a pool of 10 eggs was used as the template for cDNA synthesis, which was performed with the aid of the Improm II Reverse Transcription System kit (Promega, CA), following the manufacturer's instructions. Glycerol-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. A pair of RT-PCR primers for each pectinase gene was constructed using the Primer 3 program, version 4.0 (www.frodo.wi.mit.edu/primer3), optimizing the conditions for RT-PCR: PME_RT-PCR-Forward 5'-GACCGCAAAAACGACGAA-3'; PME_RT-PCR-Reverse 5'-GTGCTTGGTCTCTTCCA-3'; endo-PG_RT-PCR-Forward 5'-TTGACTGATTGGACCGTTG-3'; and endo-PG_RT-PCR-Reverse 5'-CACCATCAAACACGCAGTAG-3'. The reactions were conducted in a Pico Real-Time PCR System (Illumina), and the reaction cycle was 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 40 s. The comparative 2^{-ΔCT} method described by Schmittgen and Livak (2008) was used to analyze the transcripts levels. CT values were determined in triplicate and each experiment (development stages or tissues) included a negative control (reaction without template).

Immunodetection of Pectinases by Western Blotting. Polyclonal antibodies against the pectinases were produced using recombinant pectinases expressed in the yeast *Pichia pastoris*. cDNA containing the SI-pectinases open reading frames (ORFs), excluding the coding

sequence for the signal peptide, was obtained by PCR using the following primers containing sites (underlined) for the selected restriction enzymes: PME_Forward: 5'-AAACTGCAGGAAACCAAAATCCA CCAGG-3' (Pst I), PME_Reverse: 5'-AAAAGCGGCCCGCTGCTCC ACTTCCGGTATTGGC-3' (Not I), endo-PG_Forward: 5'-GGAATTC ACCCCTGCAGTAGGAGCGAG-3' (Eco RI), endo-PG_Reverse: AAAAGCGGCCGCACAAGAAAATCCGGATGG-3' (Not I). The PCR products were purified, digested with restriction enzymes, and ligated into the expression vectors pPICZαB and pPICZαA (Invitrogen, CA) for SI-PME and SI-endoPG, respectively. The recombinant plasmids were linearized with the *Pme* I restriction enzyme and used to transform competent *P. pastoris* (KM71H) by electroporation. The recombinant clones were selected, grown, and induced to expression following the manual for the EasySelect Pichia Expression Kit (Invitrogen, CA). The recombinant proteins in the supernatant were purified by affinity chromatography in a nickel column (Ni-NTA superflow, Qiagen Inc., CA).

Antibodies were raised in Swiss albino mice using the purified recombinant SI-PME and SI-endoPG. Protein extract samples for Western blotting were obtained from the same tissues analyzed by qRT-PCR. This material was individually extracted with the Trizol reagent and quantified with the aid of a BCA kit (Thermo Scientific, CA) following the manufacturer's instructions.

Approximately 4 μg of insect protein extracts and 0.25 μg of the purified recombinant enzyme (positive control) were used in Western blotting. The samples were separated for 2 h at 150 mA in 12% SDS-PAGE and then transferred to a PVDF membrane by electroblotting in transfer buffer (200 mM of Tris-HCl, 50 mM of glycine and 20% methanol). The membrane was incubated overnight in a blocking solution containing 5% defatted milk in TBS (50 mM of Tris-HCl, pH 8.0, 150 mM of NaCl) and washed with 1X TBS. Next, the membrane was incubated for 2 h with the primary specific antibody raised in mice (1: 2,500), washed in TBS, and then incubated for 90 min with the secondary antibody Anti-Mouse IgG conjugated with the alkaline phosphatase (Sigma-Aldrich Inc., MO) (1:1,000), washed with 1X TBS, and revealed with the NBT-BCIP (Thermo Scientific Pierce, IL) substrate for alkaline phosphatase.

Genomic DNA Analysis. Genomic DNA was extracted from the fat body of *S. levis* larvae to investigate the presence of introns. To prevent contamination from an exogenous source (especially endosymbionts in the gut), the portion of this tissue that was not in direct contact with the integument and midgut was used.

Approximately 2–3 mg of tissue was solubilized in 700 μl of saline buffer (150 mM of NaCl, 25 mM of ethylenediaminetetraacetic acid, 0.2% SDS) and treated with 15 μl of proteinase K (10 mg/ml) followed by 5 h of incubation at 60°C and 300 rpm. In total, 400 μl of phenol:chloroform:isoamyl alcohol (24:24:1) was added to the resulting solution, and this mixture was incubated at 37°C and 300 rpm for 30 min. After 5 min of centrifugation at 15,000 × g, the supernatant was transferred to a new tube and nucleic acid was precipitated with ethanol, dried, resuspended in 0.1 mg/ml of RNase, and kept at 37°C for 30 min. DNA was quantified using a NanoDrop spectrophotometer, and integrity was verified by 1% agarose gel electrophoresis. To certify that the DNA samples were indeed free of contamination by endosymbiotic DNA, a simple diagnosis test was performed by PCR using 16S and internal transcribed spacer (ITS) ribosomal primers. ITS1 and ITS4 (Woo et al. 2010) and 16S_338F and 16S_1492R (Piterina et al. 2010) primers were used to amplify DNA extracted from the fat body, genomic DNA samples extracted from the gut (as the positive control), and a reaction without DNA (as the negative control). For ITS analysis, genomic DNA extracted from the yeast *P. pastoris* was used as an extra positive control. PCRs were carried out using 50 ng of the DNA, 0.2 mM of dNTPs (Invitrogen, CA), 1 × PCR buffer (20 mM of Tris HCl, pH 8.4, 1.5 mM of MgCl₂ and 50 mM of KCl), 0.4 μM of each primer, 1U of Taq DNA polymerase (Invitrogen, CA), and milli-Q water for a final volume of 25 μl. The PCR cycling conditions were

94°C for 8 min, followed by 35 cycles of 1 min at 94°C, 45 s at 51°C, 5 min at 72°C, and a final step of 10 min at 72°C. The fragments were analyzed in a 1% agarose gel for the presence or absence of amplicons for the 16S region and the size of amplicons in the ITS region.

The presence of introns in the pectinase genes was verified by the amplification of the genes using different combinations of the primers employed for cloning in a pPICZ α vector and qRT-PCR. cDNA synthesized from RNA from 20-d-old larvae was used as nonintron control. PCR was conducted as described above. The amplicons were analyzed in a 1.5% agarose gel stained with ethidium bromide. Genomic fragments were excised from the gel, purified, sequenced, and compared with the respective ORFs.

Results and Discussion

Characterization of SI-Pectinase Sequences. The full-length ORF of SI-PME is composed of 1,158 bp coding a 386-amino acid polypeptide, including a putative 16-residue signal peptide. SI-endoPG exhibited 1,092 bp coding a 364-amino acid polypeptide, also including a putative signal peptide of 19 residues (Fig. 1). The analysis of mature amino acid sequences, excluding the peptide signal sequence, revealed a predicted 40 kDa for SI-PME, with an isoelectric point of 6.83 and a single N-glycosylation site (⁵⁴NHTD⁵⁷) but no O-glycosylation site. The same analysis for the SI-endoPG amino acid sequence revealed a predicted 36-kDa protein with an isoelectric point of 8.77, four N-glycosylation sites (¹⁸²NSSG¹⁸⁵, ²¹²NLTC²¹⁵, ²³⁹NVTF²⁴¹, and ³⁴⁰NWSG³⁴³), and no O-glycosylation site (Fig. 1). The presence of a signal peptide at the N-terminus of both *S. levis* pectinases and glycosylation sites indicates that these are secreted proteins. The features were verified in PCWDEs from other insects (Pauchet et al. 2010), as expected for digestive enzymes.

Inference of the Occurrence of Insect Pectinases. The alignment showed in Supp Fig. S1 (online only) demonstrates the high similarity between *S. levis* and PME from other insects, such as the *Si. oryzae* and *Dendroctonus ponderosae*. These three species are phytophagous and exhibit similar feeding behavior in the larval stage (Cerdeja et al. 1999, Reddy et al. 2002, Keeling et al. 2012), suggesting that insect PMEs share a similar physiological role. SI-endoPG demonstrated high

similarity with EndoPGs from *Si. oryzae*, *D. ponderosae*, *Chrysomela tremulae*, *Gastrophysa viridula*, *Leptinotarsa decemli*, and *Phaedon cochleariae*. These species are from the infraorder Cucujiformia, which includes a large number of efficient herbivorous insects (Pauchet et al. 2010). It is believed that pectin content and its degree of methylation are involved in resistance to insect attacks (Dreyer and Campbell 1987). Considering the importance of pectinases in the invasion mechanism of microorganisms in hosts, it is possible that insect pectinases are directly associated with herbivory. For instance, Ma et al. (1990) suggest that some insects, such as aphids, which have piercing-sucking mouthparts and use pectinases in the saliva to facilitate penetration into the host plant.

The alignment showed in Supp Fig. S2 (online only) demonstrates the high similarity among insect endo-PGs and, particularly, that the insect group is closely related to the fungal group. Indeed, several amino acid residues are common in all insect and fungal sequences but are not found in bacteria or plant sequences. An extended N-terminus has been found only in bacterial and plant endo-PGs. Studying 43 homologous sequences, Cho et al. (2001) found this extension in all 17 plants and five bacteria analyzed, whereas all 21 fungal endo-PGs lacked this feature. In addition, although less frequent, a long C-terminal extension was also only found in bacteria and plants but not in fungi, except *Claviceps purpurea* (Van Santen et al. 1999, Cho et al. 2001).

Another feature shared only by insects and fungi is the presence of eight conserved cysteine residues distributed along the primary structure of the enzyme responsible for the formation of four disulfide bonds (Cys²⁷-Cys⁴², Cys¹⁹⁷-Cys²¹⁵, Cys³³¹-Cys³³⁶, and Cys³⁵⁵-Cys³⁶⁴) (Van Santen et al. 1999, Cho et al. 2001). Bacterial and plant endo-PGs may also have disulfide bonds, but the cysteine residues positions are not conserved as in insects and fungi (Pickersgill et al. 1998, Cho et al. 2001). Except in *Ph. cochleariae*, all insect endo-PGs have two extra cysteine conserved residues (Cys¹⁴² and Cys¹⁴⁶) that enable other disulfide bonds (Pauchet et al. 2010). These exclusive two extra cysteine conserved residues in the insect group strongly indicate that endo-PG sequences are not derived from endosymbiont contamination and, therefore, are indeed derived from the insect genomes.

SI-PME	1	MKIIVLLPVLVALACANQNPPGTSSRPILTASEANYITKEYLQGWSPPS	50
	51	I ST NHTDY TVGGGGYSTIQAAVNDAINAGGSNRKYIKINTGTYYQVVYIP	100
	101	NTNVPLTIYGAGSRPDNTVITLNMPAQTPSAYKNLVNPNDAFFKPGDPA	150
	151	YSIYNGCASSSGTIGTSCSTVFWVLAPNVQIVNLQIQNSAKNKGDDQQA	200
	201	LQTNSDRIQVHNVLGHQDTLCAGSGGTDQIAHYNTYIEGDIIDYVFG	250
	251	GGTAIFESCIFYSKADRNDESIVIFAPDTPHQMYGYLVIKSTITGDSAW	300
	301	SSSKKVYLGRSWDAGVKSANAYVPGTSPNGQLVIRETTINGIVPNSAPWT	350
	351	TATSGRAYAGNAANSRDLNQNFRNFWYANTGSGA	386
	SI-EndoPG	1	MNLLVASLLLVLVARAAYA TPAVGASCTVSSYNDVAGAVSSCTSITLKG
51		TVPAGKALIMKLKSGTTVKVAGTIKFAVSEWKGPLVEISGSKITFSGAGG	100
101		YFDGQGASYWDGQGDGKTKPKFFRIKTTGGSHFNLIKLLNCPHQCVSIN	150
151		PASDTTLTDWTVDVSAGDSKGGKNTDGFDLSSSSGITIKHAVVKNQDDCV	200
201		AVNQGSYHYVFN <u>QLTCS</u> SGGHGLSLSVGGSSQNGNPNTVK <u>NVTF</u> SDCTVTNS	250
251		RNGIHVKTHNDAGTGAISDVTYKNIKLSGITNYGINIQEDYENGGSSGNP	300
301		KANIPISKLNLSVTSMSGGSSMPVYILCGSNGCANN <u>NWSG</u> VSI GNGK	350
351		KKNSCNYKPSGFSC	364

Fig. 1. Predicted primary structure of SI-pectinases. Deduced amino acid sequences of SI-PME and SI-endoPG. Residues in bold indicate putative peptide signals for extracellular secretion and underlined residues refer to probable glycosylation sites.

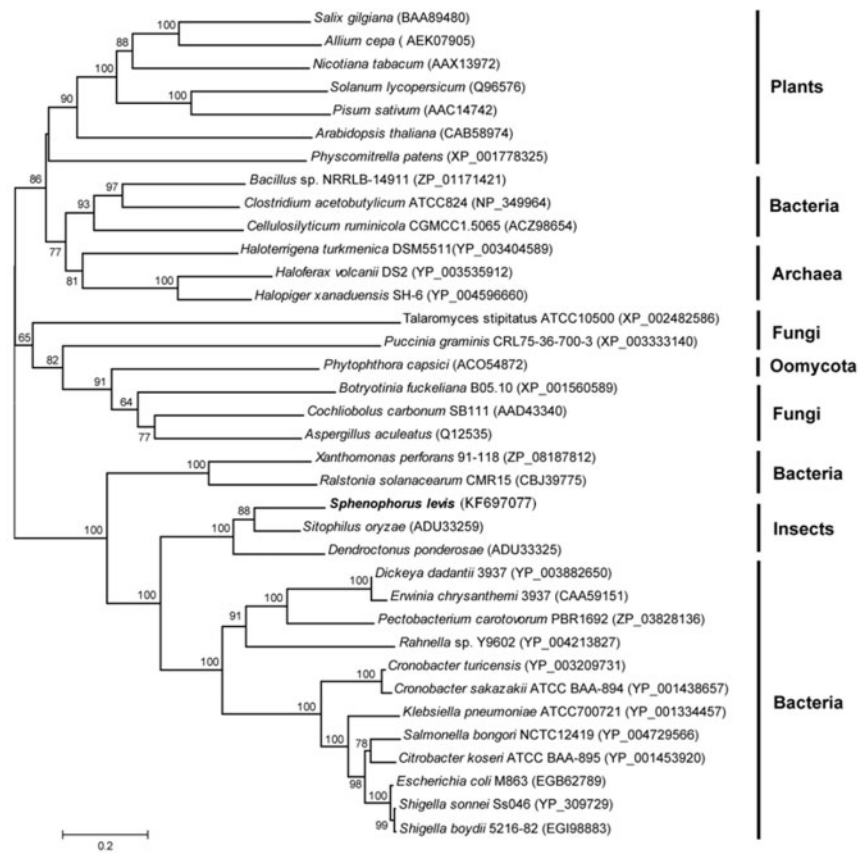


Fig. 2. Phylogenetic tree of PMEs. Phylogenetic reconstruction of 36 PMEs from plants, fungi, bacteria, archaea, and insects conducted using neighbor-joining method. Numbers in branches indicate bootstrap percentage values after 1,000 replicates. GenBank accession numbers are shown adjacent to each enzyme and *S. levis* pectinase is highlighted in bold. The bar scale (left bottom) shows percentage of dissimilarity among sequences.

Figure 2 displays the SI-PME tree inferred from the amino acid sequences of PMEs from several different organisms. This analysis suggests that bacterial PMEs, unlike what occurs with most of the taxa, did not originate from a unique episode of genetic introgression, demonstrating a polyphyletic origin with at least three different emergence events. Moreover, the results show that insect PMEs constitute a single clade among two splitting bacterial groups, suggesting a close phylogenetic relationship between the PMEs of insects and bacteria.

Figure 3 displays the SI-endoPG tree inferred from amino acid sequences of endo-PGs from several different organisms. This phylogenetic analysis reveals that insect endo-PGs form a cohesive group associated with two fungal sister groups (ascomycetes and basidiomycetes). However, the endo-PGs from insects are closer to those of ascomycetes than basidiomycetes. Thus, insect endo-PGs may have originated from ascomycete endo-PGs through a horizontal transfer event.

The first phylogenetic studies on insect pectinases were performed in *Si. oryzae* by Shen et al. (2003, 2005). The authors identified a PME and an endo-PG by Southern blotting using DNA extracted from the sterile legs of the beetle and demonstrated that these genes are indeed integrated into the insect genome. The authors also verified the close proximity between the insect and bacterial PME as well as the close proximity between insect and fungal endo-PG. Neither PME nor endo-PG activity have been identified in primitive animal taxa, suggesting a horizontal gene transfer origin from bacteria for PME and from fungi for endo-PG. The same phylogenetic relationship has been found for three endo-PGs from *Lygus lineolaris* (Allen and Mertens 2008). Therefore, the present results, which are supported by a larger, more diverse amount of analyzed sequences, furnish evidence corroborating

the theory regarding the origin of PME and endo-PG in insects through horizontal transfer events.

Investigation of Introns in SI-Pectinase Genes. To investigate possible introns in SI-pectinases and clarify their prokaryotic or eukaryotic origin, the genes were amplified from the genomic DNA extracted from the fat body (Supp Item 1 and Fig. S3 [online only]) and compared with the respective amplicons obtained from cDNA. The amplicons obtained from both cDNA SI-PME and genomic SI-PME (KF697075) had the same length, excluding the presence of introns (Fig. 4B). Differences between genomic amplicons and cDNA amplicons were observed for SI-endoPG (Fig. 4B). Primer combination 3 generated an expected fragment with 100 bp from the cDNA sample and an ~250-bp fragment for the genomic sample, indicating the presence of an intron of ~150 bp. The amplicons generated using primer combination 4 also exhibited a difference in size, since the fragment obtained from genomic DNA (KF697076) was ~800 bp and the cDNA fragment was about 600 bp. Therefore, two introns were expected for the region flanked by primer combination 4: one of about 150 bp evidenced by combination 3 and a smaller one close to 50 bp.

The presence of the two deduced introns was confirmed by the sequencing of these genomic fragments (Fig. 4C). The two introns in the coding sequence of SI-endoPG had 166 bp (509–675 bp) and 53 bp (1,003–1,056 bp), respectively. These introns exhibited the typical 5'-GT at the beginning and a 3'-AG at the end (Wu and Krainer 1999) (Fig. 4C). As is well known, introns are very rare in prokaryotic organisms in contrast to fungi, in which introns are quite common. Therefore, the presence of introns in the SI-endoPG gene, but not in the SI-PME gene is additional evidence supporting the theory regarding the origin of insect pectinases.

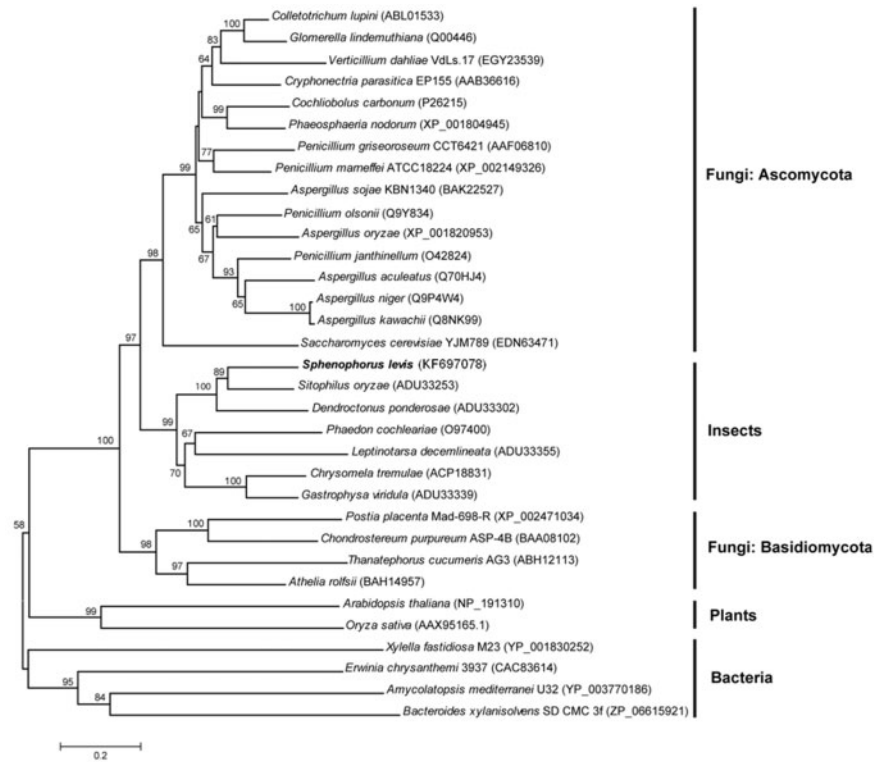


Fig. 3. Phylogenetic analysis of endo-PGs. Phylogenetic reconstruction of 34 polygalacturonases from plants, fungi, bacteria, and insects performed using neighbor-joining method. Numbers in branches indicate bootstrap percentage values after 1,000 replicates. GenBank accession numbers are shown adjacent to each enzyme and *S. levis* is in bold.

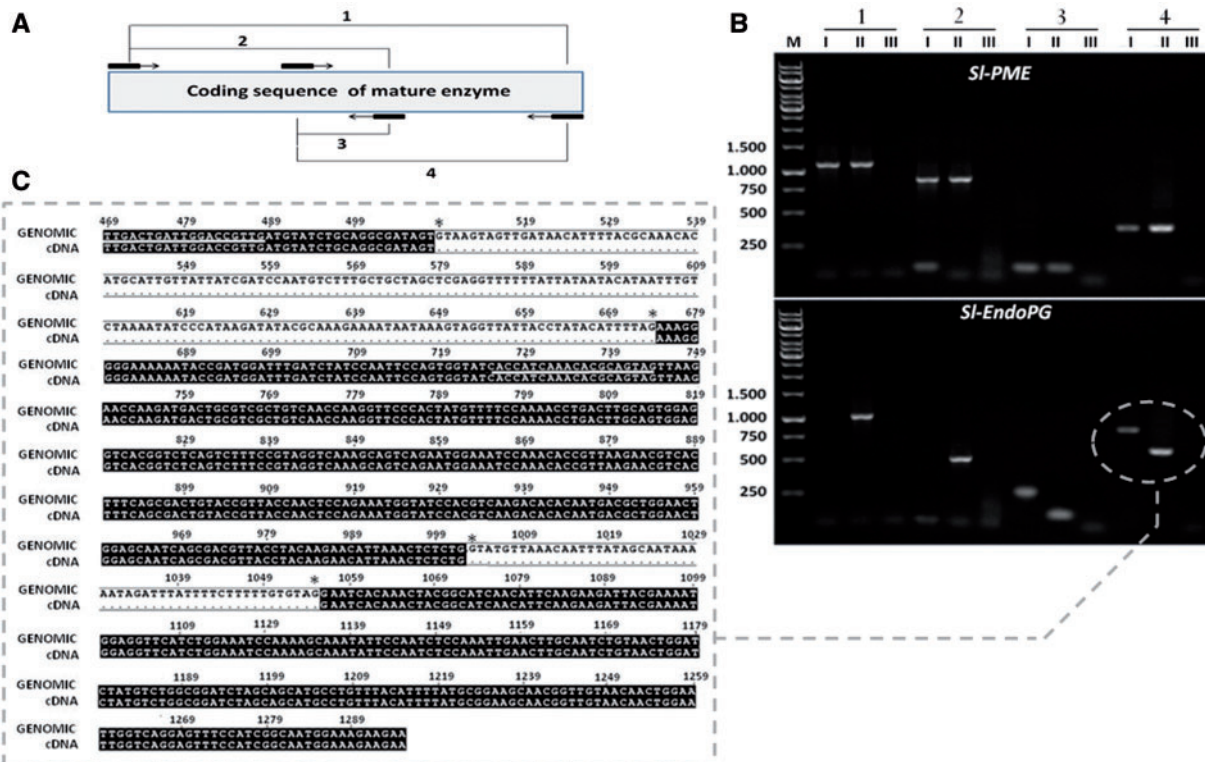


Fig. 4. Pectinase fragments amplified from *S. levis* cDNA and genomic DNA. PCR conducted with primers used for cloning ORFs in pPICZ α vectors and primers for qRT-PCR analysis. (A) Representative scheme of primer position on coding sequence of mature SI-pectinases. (B) Gel separation of fragments generated by PCR; M, GeneRuler ladder 1 kb (Thermo Scientific Fermentas, CA); 1–4, primer combinations; I, reaction with genomic DNA template; II, reaction with cDNA template; III, negative control. (C) Alignment between SI-endoPG sequences from both genomic and cDNA samples, showing two small introns. Sequences underlined in white indicate forward and reverse primers used for sequencing of genomic SI-endoPG. Asterisks indicate 5' and 3' of two introns identified.

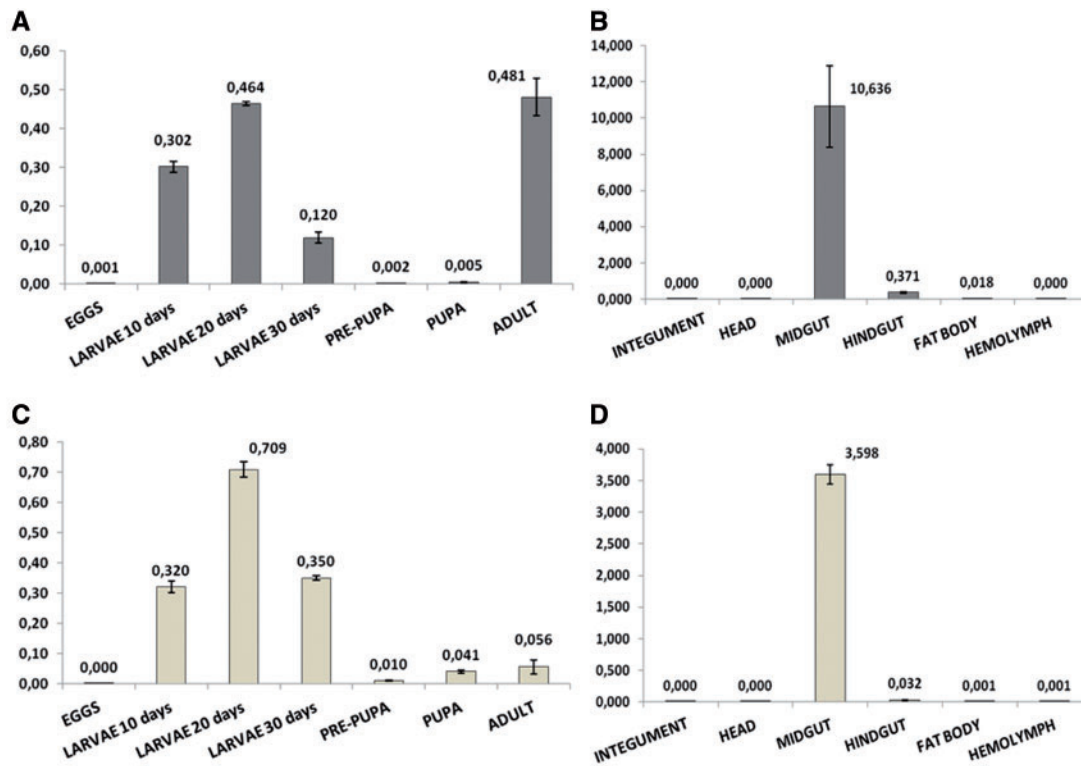


Fig. 5. Analysis of SI-pectinase gene expression. Expression analysis of SI-PME and SI-endoPG genes carried out by qRT-PCR. Values represent relationship between transcription levels of target gene and GAPDH gene. (A) SI-PME gene expression in different stages of development. (B) SI-PME gene expression in different tissues of 30-d-old larvae. (C) SI-endoPG gene expression in different stages of development. (D) SI-endoPG gene expression in different tissues of 30-d-old larvae.

mRNA Expression Analysis. To improve the understanding of the physiological role of *S. levis* pectinases, seven developmental stages and tissues from 30-d-old larvae were analyzed by qRT-PCR. Both gene transcript levels were estimated by comparison to transcript levels of the GAPDH gene. Figure 5A and C show the expression pattern of the SI-PME and SI-endoPG gene transcripts, respectively, throughout the lifecycle of the insect. The highest SI-PME expression was found in adults (0.481), followed by the larval stages, in which high transcript levels were detected. In contrast, very low transcripts levels were found in eggs as well as in the prepupal and pupal stages, suggesting a probable basal transcription level. The highest SI-endoPG transcript levels were detected in the larval stage, with a peak (0.708) in 20-d-old larvae, followed by the 30-d-old larvae (0.350) and the 10-d-old larvae (0.320). Once again, low expression was found in eggs and prepupae. Moreover, similar values were found in pupae and adults, which were much lower than in the larval stages.

Both SI-PME and SI-endoPG transcripts are detected in feeding stages, which is expected for digestive enzymes. A similar pattern has been observed for the main digestive enzyme in *S. levis* larvae—a cathepsin L cysteine peptidase (SI-CathL) (Fonseca et al. 2012). Different levels of SI-pectinase gene expression were observed between larvae and adults. The predominance of pectinase mRNAs in the larval stage may be explained by different feeding habits and plant attack mechanisms, as adult insects use the rostrum to suck sugarcane sap instead of opening galleries as larvae do (Fonseca et al. 2012). The reasons for SI-PME transcript detection in adults are not clear, but, as all adult insects analyzed were females, SI-PME may be related to the invasion process and digging a cavity for egg deposition, since prior action of PME increases the endo-PG activity in methylated substrates.

On the other hand, the highest transcript levels (10.636 for SI-PME and 3.598 for SI-endoPG) were found in the midgut (Fig. 5B and D). No significant transcript levels were detected in other tissues for either gene, which is similar to the pattern seen in the study with

SI-CathL (Fonseca et al. 2012), again suggesting a digestive role for the enzymes.

Sugarcane is one of the major natural supplies of soluble sugars, such as sucrose, glucose, and fructose. These carbohydrates are easily assimilated by cells as an energy source because they are simple molecules the metabolism of which requires a lower level of energy, unlike the pectin complex, which is an insoluble long-chain molecule. In this context, it would be energetically infeasible for the insect to use pectinases to obtain energy from pectin in a diet rich in easily assimilated sugars. Moreover, no exo-polygalacturonases (Exo-PG) sequences were found in the cDNA library for *S. levis*. In fact, to the best of our knowledge, no Exo-PG has been identified in insects. Exo-PG and endo-PG catalyze the hydrolysis of α -1,4 glycosidic bonds between galacturonic acid monomers and also have enhanced activity after the prior action of PME (Crelier et al. 2001). However, Exo-PG acts sequentially on terminal monomers of the chain, releasing them as products (Kashyap et al. 2001). It is plausible to infer that endo-PG is more efficient at disrupting the main pectin chain than creating free sugar monomers as an energy source. Therefore, we suggest that SI-pectinases are accessory digestive enzymes, the role of which is directly related to the disorganization of the cell wall to gain access to intracellular nutrients in the plant. The synergic PME and endo-PG activities could contribute to the breakdown of the cell, allowing access to the intracellular content by other digestive enzymes.

Immunodetection of *S. levis* Pectinases by Western Blotting.

Western blotting was performed using the same tissues analyzed by qRT-PCR to determine whether mRNA produced in the midgut indeed promotes SI-pectinase synthesis and to detect the tissues in which these proteins are present. Native SI-PME was detected in the midgut and hindgut tissues, with molecular mass of about 45 kDa (Fig. 6). The slight difference in molecular mass between the native SI-PME and recombinant SI-PME (which has ~50 kDa) is explained by extra residues at the C-terminus (15 residues from the c-myc epitope and 6

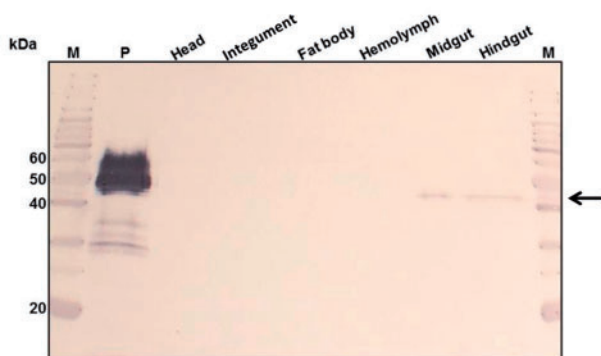


Fig. 6. Immunodetection of SI-PME in tissues of *S. levis* larvae. Immunodetection of SI-PME performed by Western blotting using polyclonal antibodies. M, Benchmark molecular weight marker (Invitrogen, CA); P, purified recombinant SI-PME, head→hindgut, total protein extracted from indicated tissues.

residues of His-tag) added from the expression vector and the glycosylation of recombinant SI-PME by *P. pastoris*. The synthesis of SI-PME mRNA in the midgut was expected, but no gene expression was detected in the hindgut, which suggests that this protein is carried to the hindgut during the digestion process. The same analysis was conducted for SI-endoPG (data not shown), but the native enzyme was not detected, probably due to low specificity of the polyclonal antibody generated against it. However, considering the SI-endoPG expression in the midgut and the fact these two pectinases act synergistically, it is probable that SI-endoPG also acts in the midgut.

The present results provide evidence that SI-pectinases are indeed integrated into the *S. levis* genome and corroborate the hypothesis put forth by Shen et al. on horizontal gene transfer for insect pectinases. SI-pectinases are expressed in all feeding stages, possibly as important accessory digestive enzymes acting in the midgut and contributing to the efficient herbivory of *S. levis*. Therefore, we suggest these enzymes as future targets for the control of this insect in sugarcane plantations.

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