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Comparative analysis of expression profiling of the trypsin and chymotrypsin genes from Lepidoptera species with different levels of sensitivity to soybean peptidase inhibitors

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ABSTRACT

Peptidase inhibitors (PIs) are essential proteins involved in plant resistance to herbivorous insects, yet many insect species are able to escape the negative effects of these molecules. We compared the effects of acute and chronic ingestion of soybean peptidase inhibitors (SPIs) on *Spodoptera frugiperda* and *Diatraea saccharalis*, two Lepidoptera species with different sensitivities to SPI ingestion. We analyzed the trypsin and chymotrypsin gene expression profiles in both species. Acute exposure of *S. frugiperda* to the inhibitors activated seven genes (SfChy5, SfChy9, SfChy19, SfChy22, SfTry6, SfTry8, and SfTry10), whereas chronic exposure activated 16 genes (SfChy2, SfChy4, SfChy5, SfChy8, SfChy9, SfChy11, SfChy12, SfChy15, SfChy17, SfChy21, SfChy22, SfTry6, SfTry8, SfTry9, SfTry10, and SfTry12). By contrast, the challenge of *D. saccharalis* with SPIs did not differentially induce the expression of trypsin- or chymotrypsin-encoding genes, with the exception of DsChy7. Bayesian phylogenetic analysis of *S. frugiperda* trypsin protein sequences revealed two gene clades: one composed of genes responsive to the SPIs and a second composed of the unresponsive genes. *D. saccharalis* trypsin proteins were clustered nearest to the *S. frugiperda* unresponsive genes. Overall, our findings support a hypothesized mechanism of resistance of Noctuidae moths to SPIs, involving gene number expansion of trypsin and chymotrypsin families and regulation of gene expression, which could also explain the variable susceptibility between *S. frugiperda* and *D. saccharalis* to these plant inhibitors.

1. Introduction

Insect herbivores cause decreased production in several crops around the world. These pests have been controlled by the widespread use of insecticides, causing damage to not only human health but also the environment. This situation clearly requires the development of new methods that can control the insects without causing further damage. One promising pest control strategy encompasses the use of plant peptidase inhibitors (PIs) (Bode et al., 2013; Boulter, 1993; Bown et al., 1997; Murdock and Shade, 2002; Prasad et al., 2010; Ryan, 1990; Srinivasan et al., 2005).

The defense capability of plant PIs against insects was first reported by Green and Ryan (1972). These authors demonstrated an induction and accumulation of these inhibitors in the shoots of tomato and potato plants after injury caused by insects. The plant PIs acted as pseudo-substrates specific for insect digestive peptidases, resulting in the formation of a stable complex that limited the proteolysis reaction (Tiffin and Gaut, 2001). Studies have extensively characterized these inhibitors from various plant species (Damle et al., 2005; Hartl et al., 2010; Lomate and Hivrale, 2012; Singh et al., 2014; Steppuhn and Baldwin, 2007).

Although plant PIs are efficient in inhibiting insect digestive peptidases, many authors have shown that insects can develop strategies to overcome the effects of the inhibitors. These strategies involve the activity of digestive enzymes and gene modulation, mainly of serine endopeptidases. Serine endopeptidases are present in the midgut of almost all insect orders and are the most abundant peptidases (Terra and Ferreira, 1994). Studies of the deleterious effect of PIs on the midguts of insects have mainly considered two serine endopeptidases: trypsin and chymotrypsin. *Spodoptera exigua* (Lepidoptera, Noctuidae) adapts to the presence of the potato proteinase inhibitor II effect through the synthesis of insensitive trypsins (Jongsma et al., 1995); *Helicoverpa armigera* (Lepidoptera, Noctuidae) secretes peptidases that hydrolyze the PIs (Giri et al., 1998) and expresses peptidases with

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altered substrate specificity (Wu et al., 1997); *Chilo suppressalis* (Lepidoptera, Crambidae), trypsin and chymotrypsin genes showed different transcript levels in insects that feed on different host plants (Ge et al., 2012).

Spodoptera frugiperda (Lepidoptera, Noctuidae) is one of the major Lepidoptera pests. This species feeds on a wide range of crops, resulting in severe yield losses. Thus, understanding the mechanism by which the larvae overcome the effects of plant PIs is important for defining strategies to control this pest. It has been noted that the chronic ingestion of soybean peptidase inhibitors (SPIs) does not affect *S. frugiperda*. According to Paulillo et al. (2000), the resistance mechanism of the fall armyworm; i.e., *S. frugiperda*, to SPIs is related to changes in the complement of proteolytic enzymes in the insect midgut. Other authors studying gene expression patterns of serine endopeptidases showed the occurrence of *de novo* synthesis and/or the upregulation of existing genes after a PI challenge (Brioschi et al., 2007; Oliveira et al., 2013). By contrast, in the sugarcane borer *Diatraea saccharalis* (Lepidoptera, Crambidae), which is a more specialist insect, SPIs exerted antinutritional activity on the growth of neonate larvae (Pompermayer et al., 2001). Adult deformation and anti-metabolic effects causing a reduction in larval weight in *D. saccharalis* have also been reported as the result of exposure to trypsin inhibitor from *Theobroma cacao* and *Inga laurina* (Paulillo et al., 2012; Ramos et al., 2012).

However, the molecular mechanisms involved in the variable susceptibility of the Lepidoptera species to SPIs remain unknown.

In this study, we investigated the role of the midgut serine endopeptidases in the responses of different Lepidoptera species to soybean peptidase inhibitors. We studied two species for this purpose: one that is less susceptible (*S. frugiperda*) and one that is more susceptible (*D. saccharalis*) to these inhibitors. The results provide new insights to understanding the variable susceptibility of these two Lepidoptera species.

1.1. Experimental procedures

1.1.1. Serine endopeptidase identification

Trypsin and chymotrypsin protein sequences from *S. frugiperda* were retrieved from Dias et al. (2015) and residues were numbered based on bovine chymotrypsinogen.

For trypsin and chymotrypsin protein sequences from sugarcane borer, the identification was performed on separate sets of 50 larvae each with three replicates of each set on an artificial diet, which was adapted from that of King and Hartley (1985), supplemented either with or without SPI (0.5% w/v). Total RNA was isolated from insect gut tissues using TRIzol reagent (Invitrogen). Sequencing was conducted by Fasteris SA (Geneva, Switzerland) following the manufacturer's instructions (Illumina, San Diego, CA). Illumina reads were processed using the Illumina pipeline version 1.8 or later. Prior to assembly, raw FASTQ (Illumina) data were subjected to filtering using the SeqyClean pipeline (https://bitbucket.org/izhbannikov/seqyclean/) to remove low-quality bases (score < 25), poly-A|T tails and adapters. The Illumina data were normalized by applying single-pass digital normalization using the normalize_by_kmer_coverage procedure of the Trinity assembler (Haas et al., 2013). Then, a *de novo* assembly was generated using the MIRA assembler (Chevreux et al., 2004) with two passes and the following settings: (i) spoiler detection on (−AS:sd = yes), (ii) 70% relative percentage of exact word matches (−SK:pr = 70), (iii) maximum megahub ratio = 1 (−SK:mmhr = 1) and (iv) stepping increment = 2 (−SK:hss = 2). Additionally, Illumina technology-specific settings included: (i) quality clipping on (−CL:qc = yes), (ii) minimum base quality = 5 (−CL:qcmq = 5), (iii) length of window for quality clipping = 5 (−CL:qcwl = 5) and (iv) elimination of sequences that form a contig with <3 reads (−AS:mrpc = 3). Contigs with <200 bases and <7× coverage were filtered out of the Common Assembly Format (CAF) file generated by MIRA.

The sequences were verified to contain the catalytic triad residues (His57, Asp102 and Ser195) and the zymogen activation motif. Moreover, the presence of Asp189 was used to determine trypsin specificity (Huber and Bode, 1977). The new *D. saccharalis* sequences have been submitted to NCBI with the following GenBank accession numbers: DsTry1 - **KR024669**, DsTry2 - **R024670**, DsTry3 - **KR024671**, DsTry5 - **KR024672**, DsChy1 - **KR024673**, DsChy2 - **KR024674**, DsChy3 - **KR024675**, DsChy4 - **KR024676**, DsChy5 - **KR024677**, DsChy6 - **R024678**, DsChy7 - **KR024679**, DsChy8 - **KR024680**, DsChy9 - **KR024681**.

The enzyme subsite classification was based on Schechter and Berger (1968), in which these regions were named according to the substrate residues in contact with the enzyme binding site. The residues in the substrate were numbered in ascending order from the cleavage point as P1, P2, …, Pn from the C- to N-terminal and as P1′, P2′, …, Pn′ from the N- to C-terminal region. In the enzyme, all residues contacted by the residue P1 were called subsite S1, and the residues contacted by the substrate residue P2 were called subsite S2, *etc*. The subsite amino acid composition was based on Hedstrom (2002).

1.1.2. Protein sequence analysis

To analyze the evolutionary relatedness among *S. frugiperda* and *D. saccharalis* trypsin and chymotrypsin protein sequences, molecular phylogenetic analyses were conducted. For this process, all amino acid sequences were aligned using Muscle software (Edgar, 2004). The phylogenetic analyses were conducted by Bayesian inference using MrBayes software (version 3.2.1 mpi-enable) (Ronquist and Huelsenbeck, 2003). The amino acid substitution models were chosen using ProtTest (version 3) (Abascal et al., 2005) with the Akaike Information Criterion (AIC) parameter. A total of 5×10^6 generations were run and Markov Chains were sampled every 100 generations. A total of 25% of the initial trees were removed as "burn in" to ensure sampling of the trees after topology convergence. The resulting tree was organized and analyzed using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

1.1.3. Insect rearing

Each experiment consisted of biological triplicates, 20 neonates for each repeat in each treatment, reared on either artificial diet with or without SPIs (0.5% w/v) and the bioassays were repeated three times. The larvae were reared under a photoperiod of 14:10 (L:D) h at 25 ± 1 °C and 70 ± 10% relative humidity. The artificial diet for *S. frugiperda* was prepared as described by Greene et al. (1976), and the artificial diet for *D. saccharalis* was prepared according to King and Hartley (1985).

In the SPI chronic ingestion experiment, *S. frugiperda* neonate larvae were reared on an artificial diet with or without SPIs (0.5% w/v) until the 6th instar (last-instar). For the acute assay, the larvae were reared to the 6th instar stage on a diet without SPIs. Then, the *S. frugiperda* larvae were randomly divided into two groups. The first group was fed a diet containing SPIs (0.5% w/v) for 48 h, whereas the second group received a diet without SPIs. The procedures were the same for *D. saccharalis*, but for both the acute and chronic experiments, the analysis began at the 5th instar (last-instar).

1.1.4. Extraction of the midgut, isolation of total RNA and cDNA synthesis

The larvae were anesthetized on ice prior to dissection. The larval midguts were gently removed, and the samples were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using TRIzol reagent (Invitrogen). Residual genomic DNA was removed from the RNA using RNase-free DNase (Fermentas). The RNA was analyzed on a 1% denatured agarose gel to ensure its integrity. The 260/280 nm and 260/230 nm spectrographic absorption ratios were evaluated for protein and solvent contamination. A total of 1 μg of DNA-free total RNA was converted into cDNA using a mix of random