REPORT

Identification of a new trypsin from Spodoptera frugiperda involved in a defensive mechanism against the Bacillus thuringiensis *Cry1Ca1 toxin*

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ABSTRACT

Both, insecticidal formulations based on the entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) and transgenic plants expressing *Bt* Cry toxins, have been threatened by the potential appearance of insect resistance in major crop pests. With the aim of identifying genes and mechanisms triggered against a *Bt* Cry1-class toxin in the midgut of fall armyworm *Spodoptera frugiperda*, the major pest of maize in Cuba, subtractive cDNA libraries of the molecular interaction insect-*Bt* Cry1Ca1 toxin were constructed. Among those genes specifically regulated in response to the intoxication, one coding for a new trypsin-like serin proteinase (*SfT6*) was identified. Gene function analysis using RNA interference showed *SfT6* plays a crucial role for the Cry1Ca1 toxicity against *S. frugiperda*; gene expression suppression caused a reduction of the proteolytic processing of Cry1Ca1 by the larval midgut juice and a reduced susceptibility of insects in bioassays. Our study represents the first report on *S. frugiperda* midgut genes differentially-expressed in response to Cry1Ca1 intoxication. Besides, we have identified and cloned the full-length cDNA sequence of a novel serine proteinase whose regulation is linked to the natural process of insect adaptation to *Bt* in order to survive the pathogenic process.

Keywords: Bacillus thuringiensis, *Spodoptera frugiperda*, Cry1Ca1, serine-proteinase, trypsin, RNA interference

*I*ntroduction

For decades, commercial formulations of the sporogenic bacterium *Bacillus thuringiensis* (*Bt*) and its parasporal crystal inclusions (composed mainly by the insecticidal Cry proteins) have been sprayed on economically important crops, sometimes indiscriminately, for the control of major insect pests [1]. More recently, *Bt cry* genes have been introduced into cultivable plants, particularly cotton and maize, to produce insect-resistant *Bt*-crops that in 2009 were grown on 50.4 million ha worldwide [2]. The major concern of such widespread use of *Bt* products deals with the possibility of the that developed resistance by key pest species. To date, three lepidopteran pest species have evolved substantial resistance to *Bt* sprays under field conditions [3, 4] while the first cases of field-evolved resistance to *Bt*-crops have been recently documented [5].

The *Bt*-insect interaction is a complex multi-step process ending up in larval death when it's effective. In Lepidoptera, ingested *Bt* spore and crystals reach the extremely alkaline and reducing environment of the larval midgut where spore germination is activated and crystal inclusions solubilize to inactive protoxins of 130-140 kDa in size. Then, midgut serine-proteinases transform protoxins into active toxins of 55-65 kDa after removing approximately 600 residues from the C-terminus and the first 28 N-terminal residues [6]. Activated monomers of the Cry toxins can cross the peritrophic matrix barrier to bind specific membrane receptor(s) on epithelial target cells, where further proteolysis may take place before pore-forming structures are formed by the plasma membrane inserted into to disturb the ionic and osmotic balances of the cell [7]. Finally, germinated spores swing into the insect hemolymph through the gaps in the epithelial cells barrier left by Cry-bursted cells to colonize the entire larval body causing septicemic death.

The molecular study of host – pathogen interaction can reveal important features of the pathogenic process as well as identify those components of defensive mechanisms triggered in the host. For insect-*Bt* interaction, such defensive responses could lead to insect adaptation to the entomopathogen after continuous exposures inducing a considerable resistance level in successive generations. One effective approach for identifying insect genes differentially expressed during exposure to *Bt* Cry toxins is suppression subtractive hybridization (SSH) [8]. SSH is based on an efficient subtraction step followed by a suppression step. PCR amplification allows the enrichment of the differentially expressed genes but suppression of those that are not differentially expressed. In addition, a normalization step is incorporated to equalize the abundance of individual mRNAs, in order to provide a better chance for detection of rare transcripts. Since SSH allows the isolation of differentially expressed cDNAs without a prior knowledge of their sequence, it is highly desirable for studying differential gene expression in systems where information on the genomic sequence is scarce (i.e., insects). Further, gene function studies can be accomplished *in vivo* by RNA interference where specific gene expression is suppressed or completely silenced after feeding larvae with a double-stranded RNA (dsRNA) synthesized from a large fragment of the correspondent gene [9].

In the present work, the molecular response elicited by *Bt* Cry1Ca poisoning in *Spodoptera frugiperda* (Lepidoptera: Noctuidae), the major pest of maize

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in Cuba, is investigated. Subtractive cDNA libraries enriched for *S. frugiperda* midgut genes differentially regulated during the exposure to a sublethal dose of the toxin were generated. Expression analysis of SSH clones showing a significant hit in the BLAST analysis and which reflected the distribution of molecular functions in the subtractive cDNA libraries allowed the identification of a new trypsin-like serin proteinase (*SfT6*) that was chosen for gene function analysis by RNAi. Gene expression suppression of *SfT6* decreased the pathogenicity of *Bt* Cry1Ca1 in *S. frugiperda* larvae and the protoxin activation rate by larval gut juice corroborating its crucial role for the *Bt* Cry1Ca1 toxic pathway in this important Lepidoptera.

Results and discussion

The present study aimed at identifying genes and mechanisms of the lepidopteran larval gut involved in the specific response against Cry1-class toxins of *B. thuringiensis*. To achieve this goal, we performed the molecular analysis of *S. frugiperda*-Cry1Ca1 toxin interaction and investigated the role of a new trypsin-like serine-protease (*SfT6*) differentially expressed in the larval midgut during the process of *Bt* intoxication. To this end, the following strategies were used: i) construction and characterization of subtractive cDNA libraries from the larval midgut tissue during the interaction *S. frugiperda*-Cry1Ca1, ii) cloning of the cDNA full-length sequence of a novel *S. frugiperda* trypsin, named here as *SfT6*, and its sequence analysis, iii) RNAi-mediated gene silencing of *SfT6* for function analysis through insect bioassays and *in vitro* proteolytic processing of the Cry1Ca1 protoxin.

Since a sublethal dose of 2 µg Cry1Ca1/cm^2 caused a complete inhibition of the larval growth and less than 5% mortality in *S. frugiperda* third-instar larvae [10], this toxin concentration was used for studying the early transcriptional response of midgut cells from larvae fed with Cry1Ca1. The construction of subtractive cDNA libraries enriched in larval midgut genes differentially regulated during the intoxication process yielded fragments ranging in size from 250 to 1000 base pairs (bp). The quality of subtracted cDNA libraries was validated by RT-PCR amplification of an internal fragment of the constitutively expressed *β-actin* transcript, and by hybridization with radioactive ^{32}P-labeled probes obtained from the unsubtracted cDNA pool [10]. In addition, the analysis of signal differentials between unsubtracted/subtracted cDNA hybridizations allowed the identification of 86 clones that were the most relevant according to their regulation. The DNA sequence of selected 86 clones was compared with GenBank/EMBL databases using the BLASTN/BLASTX algorithms. According to results, 23% of the cDNA fragments showed significant similarity to gene products involved in known biological processes; with disease/defense, metabolism (catalytic activities) and oxidative stress being the most frequently identified by using a Gene Ontology software.

One of the most interesting clones of the libraries according to its expression regulation, with a significant hit in the BLAST analysis, was a serine-proteinase named as *SfT6*. The full-length cDNA sequence of *SfT6* was PCR cloned by RACE. A fragment of 904 bp length was obtained that contained a 765 bp Open Reading Frame (ORF), spanning nucleotides 57 to 821. The ORF encoded a protein consisting of 254 amino acids with a calculated molecular mass for the mature protein of 25.1 kDa and isoelectric point of 8.6. Multiple alignment and phylogenetic relationship analysis of predicted amino acids for *SfT6* mature polypeptide denoted that the cloned fragment belongs to a trypsin-like serine-proteinase gene. The phylogenetic tree showed that the *S. frugiperda SfT6*-predicted polypeptide is in the same branch as trypsin-like deduced proteins from most lepidopteran annotated sequences (Figure 1A). Specific alignment of *SfT6* with partial sequences of *S. frugiperda* trypsin-like serine proteinases found in the integrative database SPODOBASE [11] yielded only a marginal identity of 20% among them. RT-PCR reactions conducted to investigate the developmental expression pattern of *SfT6* showed that this gene is transcribed during the actively feeding stages of the insect life (larvae and adult) while it is totally absent in embryonic and pupal stages (Figure 1B). The accumulation rate of *SfT6* dramatically increased during the progression from neonates to sixth larval instar suggesting an important role of this protease for food digestion during larval growth and development. However, the expression of *SfT6* was visibly affected when larvae were exposed to Cry1Ca1 toxin (Figure 1C). *SfT6* mRNA levels were shown to rapidly decline by 15 min of intoxication while the expression of other two larval trypsins (Sf1f02950-3-1 and Sf1f04740-5-1) remained invariable. With this result, we show for the first time, *S. frugiperda* can mount a defensive response to a *Bt* Cry1-class toxin based on, among others, a decreased expression in one particular serine proteinase from the midgut [12].

Considering the crucial role of midgut serine proteinases for the proteolytic activation of Cry1-class proteins during the *Bt* toxic pathway in Lepidoptera [6, 13], the specific downregulation of *SfT6* expression in *S. frugiperda* larval midgut during the early stages of Cry1Ca1 intoxication suggests a defensive mechanism adopted by the insect to survive the pathogenic process. To prove this hypothesis, we performed RNAi-mediated gene silencing in *S. frugiperda* by feeding newly molted fourth-instar larvae with double-stranded RNA (dsRNA) produced from a fragment of *SfT6* gene. As controls, two groups of insects fed with either buffer alone (10 mM Tris-Cl, pH 7.5; 10 mM EDTA) or a nonspecific dsRNA obtained from the *β-glucuronidase* (*gus*) gene from *Escherichia coli* were used. Both, insects' susceptibility to Cry1Ca1 and *in vitro* activation of Cry1Ca1 protoxins by larval midgut juice proteases, were evaluated in *SfT6*-knockdown insects and compared to insects from control groups. The efficacy of *SfT6*-knockdown was investigated 48 h after the corresponding RNAi treatment by qRT-PCR analysis on larval midgut total RNA. The results revealed that the oral administration of dsRNA-*SfT6* caused a significant reduction of *SfT6* gene expression in the midgut (approx. 90% of control), while feeding with dsRNA-*Gus* showed no effect on the *SfT6* levels, confirming the specificity of the process for the target gene [12].

The results of Cry1Ca1 toxicity are shown in Figure 2A, expressed as percentage of growth inhibition

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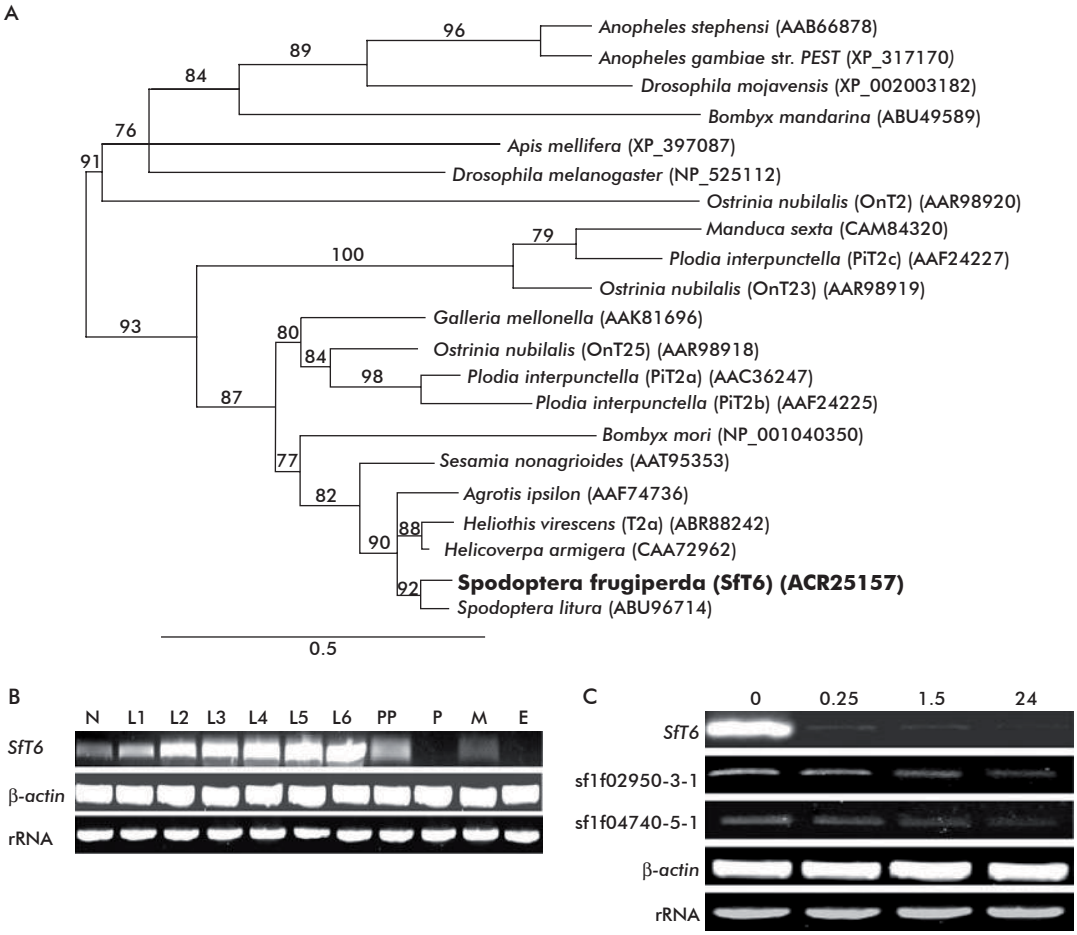

Figure 1. Phylogenetic and expression analysis of *SfT6* serine-proteinase. A) Phylogram showing the relationship between *S. frugiperda SfT6* (GeneBank Acc. Num. FJ940726) and selected insect trypsin-like proteins. Multiple sequence alignments and phylogenetic reconstructions were performed using the Phylogeny platform (http://www.phylogeny.fr/). *SfT6* is highlighted in bold. GenBank accession numbers are reported in parentheses. Bootstrap values greater than 50 are indicated along branches. The scale bar indicates the evolutionary distance. B) *SfT6* transcript accumulation at different stages of the *S. frugiperda* life cycle. N: neonates; L1-6: the six larval instars of S. frugiperda; PP: pre-pupae; P: pupae; M: moths; E: embryo. C) Expression analysis of *SfT6* and two others *S. frugiperda* trypsin genes at indicated time (h) post-intoxication with 2 μg cm^{-2} of Bt Cry1Ca1 protoxin. rRNA refers to ribosomal RNAs.

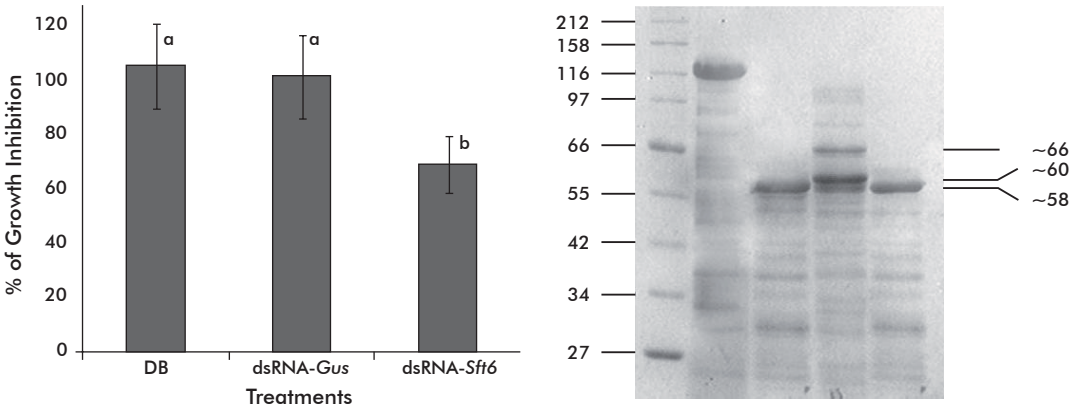

Figure 2. Function analysis of *SfT6* gene in *S. frugiperda*. A) Toxicity of Bt Cry1Ca1 protoxin (2 μg cm^{-2}) to *S. frugiperda* fourth instar larvae droplet-fed delivery buffer (DB) solutions containing 3 μg of dsRNA from either *SfT6*, gus or DB only. Bars show the means ± SE of two independent experiments. In each experiment, 24 larvae were used per treatment. The means were compared using a Kruskal–Wallis test with Dunn's post-test. The H statistic was 61.09. Treatments not sharing a common letter were significantly different ($p < 0.05$). B) SDS-12% PAGE analysis of Cry1Ca1 products from the protoxin activation process with 0.5 mg of *S. frugiperda* midgut juice proteins from larvae fed with: gus-dsRNA (lane 1), *SfT6*-dsRNA (lane 2) and buffer only (lane 3). Sizes are expressed in kDa. PT: protoxin only; MW: broad-range molecular weight marker (NEB).

(%GI) and determined in dsRNA-treated and untreated larvae 48 h after the dsRNA feeding assay. No differences in %GI values were found between larvae that were only fed with buffer (mocked) or nonspecific dsRNA-*Gus*. In contrast, larvae from the group fed with dsRNA-*SfT6* were ~30% less affected by the Cry1Ca1 protoxin in terms of %GI at the same tested concentration. On the other hand, *in vitro* protoxin processing experiments showed that after 1 h of Cry1Ca1 incubation with gut juice from larvae fed with nonspecific dsRNA-*Gus*, a single band of ~58 kDa was generated that did not differ from that obtained with gut juice from mocked (buffer-fed) larvae (Figure 2B, lanes 1 and 3). On the contrary, a moderate but clear decrease in protoxin activation rate was found when midgut juice from dsRNA-*SfT6*-fed larvae was used in the reaction (Figure 2B, lane 2). In this case, the midgut juice failed to completely process the Cry1Ca1 protoxin to the ~58 kDa product (the apparent activated form of Cry1Ca1 toxin in *S. frugiperda*) but produced in addition two major partially processed products of ~60 and ~66 kDa (Figure 2B, lane 2).

In summary, our results represent the first study on molecular defensive mechanisms triggered in the midgut cells of *S. frugiperda* larvae after intoxication with a *B. thuringiensis* Cry1-class toxin [10, 12]. Also, we have identified, cloned and characterized a novel trypsin gene from the *S. frugiperda* larval midgut that participates in a defensive mechanism elicited in response to the *Bt* Cry1Ca1 pathogenic process [12]. We have shown that the specific down-regulation of SfT6 serin-proteinase increases larval survival after exposure to Cry1Ca1 in bioassays and affects *in vitro* proteolytic processing of the protoxin in the presence of larval gut juice. Down-regulation of *SfT6* could represent a putative defensive mechanism to reduce the amounts of activated toxin monomers that can reach target gut cells, providing with a survival advantage for insects feeding on Cry1Ca1-contaminated diets. In this way, unprocessed protoxins cannot pass the peritrophic matrix and can be rapidly eliminated during the gut clearance process. Regulation of *SfT6* expression could become a target in a proteinase-mediated *Bt*-resistance mechanism. Indeed, significantly reduced mRNA levels of the *SfT6* ortholog in *Plodia interpunctella* were found in a resistant population of this insect [14].

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