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Identification, functional characterization and phylogenetic analysis of double stranded RNA degrading enzymes present in the gut of the desert locust, *Schistocerca gregaria*

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ABSTRACT

RNA interference (RNAi) has become a widely used reverse genetics tool in eukaryotes and holds great potential to contribute to the development of novel strategies for insect pest control. While previous studies clearly demonstrated that injection of dsRNA into the body cavity of the desert locust, *Schistocerca gregaria*, is highly effective to induce gene silencing effects, we observed that the RNAi response is much less sensitive to orally delivered dsRNA. In line with this, we report on the presence of a potent dsRNA degrading activity in the midgut juice. Four different *dsRNase* sequences that belong to the DNA/RNA Non-specific Nuclease superfamily were retrieved from a transcriptome database of the desert locust. Surprisingly, we have found that, in the publicly available eukaryote nucleotide sequence databases, the presence of this group of enzymes is restricted to insects and crustaceans. Nonetheless, phylogenetic analyses predict a common origin of these enzymes with the Endonuclease G (EndoG) Non-specific Nucleases that display a widespread taxonomic distribution. Moreover, in contrast to the *Sg-endoG* transcript, the four *Sg-dsRNase* transcripts appear to be specifically expressed in the gut. Finally, by means of RNAi, we provide evidence for an important contribution of dsRNase2 to the dsRNA degrading activity that is present in the gut lumen of *S. gregaria*.

1. Introduction

RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing triggered by double stranded (ds)RNA molecules. Under natural conditions, RNAi is triggered by dsRNA structures that are produced during the replication cycle of viruses or generated from repetitive elements and transposons in the cellular genome (Hammond, 2005). Thanks to its robustness and specificity, RNAi has become a widely used method to silence genes in many eukaryotic systems and may contribute to novel strategies to control agricultural pests, including a number of insect species.

Our research group has previously demonstrated that injection of dsRNA into the body cavity of the desert locust, *Schistocerca gregaria*, is a highly effective method to induce gene silencing effects (Wynant et al., 2012). Moreover, knocking down vital genes, such as *alpha-tubulin 1a* (*tubu*), generated mortality in the locust population (Wynant et al., 2012). Yet, for in field applications of RNAi-based pest control, oral delivery of the dsRNA would be of particular interest. For this purpose, one could use transgenic plants or micro-organisms that express dsRNAs targeting essential insect genes. To date, feeding of dsRNA has also been reported to induce RNAi in several insect species, including in *Bactrocera dorsalis* (Diptera) (Li et al., 2011), *Epiphyas postvittana (Lepidoptera)* (Turner et al., 2006), *Helicoverpa armigera* (Lepidoptera) (Mao et al., 2007), *Spodoptera frugiperda* (Lepidoptera) (Griebler et al., 2008), *Plutella xylostella* (*Lepidoptera*) (Bautista et al., 2009), *Manduca sexta* (Lepidoptera) (Kumar et al., 2012), *Apis mellifera* (Hymenoptera) (Hunter et al., 2010), *Rhodnius prolixus* (Hemiptera) (Araujo et al., 2006), *Acyrthosiphon pisum* (Hemiptera) (Mao and Zeng, 2012; Pitino et al., 2011), *Diabrotica virgifera* (Coleoptera) (Baum et al., 2007), *Leptinotarsa decemlineata (Coleoptera)* (Zhu et al., 2011) and *Reticulitermes flavipes* (Isoptera) (Zhou et al., 2008). Nonetheless, we show in this study that the RNAi response of the desert locust was absent ot not detectable after exposure to orally delivered dsRNA.

In the silkmoth, *Bombyx mori*, Arimatsu et al. (2007) purified a DNA/RNA non-specifc alkaline nuclease that is secreted into the midgut, where it can digest dsRNA. Therefore, it is also sometimes

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designated as a double stranded ribonuclease (dsRNase). Yet, while highest activity is displayed to dsRNA, it can also cleave ssRNA and DNA. This nuclease is characterized by the presence of a single DNA/RNA non-specific nuclease (NN)-domain that is preceded by a spacer region and a signal peptide (Arimatsu et al., 2007). Members of the DNA/RNA non-specific nuclease family have also been found in bacteria, nematodes and mammals (Low, 2003). The nematode and mammalian DNA/RNA non-specific nucleases, which are most often referred to as Endonuclease (Endo)G proteins, have been indicated to play a role in mitochondrial DNA replication and genomic DNA degradation during apoptosis (Low, 2003).

In this paper, we demonstrate the presence of potent dsRNA degradation activity in the midgut juice of the desert locust and present data on the expression in the gut of four members of a pancrustacean gene family of DNA/RNA Non-specific Nuclease (NN-)domain (Pfam) containing enzymes.

2. Materials and methods

2.1. Rearing of the desert locust, S. gregaria

Gregarious *S. gregaria* were reared under crowded conditions with controlled temperature (32 ± 1 °C), light (1 h photoperiod) and relative humidity (40–60%). They were fed daily with fresh cabbage and rolled oats. Adult locusts were developmentally synchronized by transferring them to a different cage directly after their final moult.

2.2. Retrieval of transcript sequence information from the S. gregaria transcriptome database

By using the *B. mori* alkaline nuclease transcript sequence (NM_001098274.1) as a query, transcript sequence information for *Sg-dsRNase1* (KJ135008), *Sg-dsRNase2 (KJ135009)*, *Sg-dsRNase3* (KJ135010) and *Sg-dsRNase4* (KJ135011) (KJ135011) and, by using the *Caenorhabditis elegans endonuclease (endo)G* transcript sequence (NM_058970.4), the *Sg-endoG* transcript sequence was retrieved from a transcriptome database of *S. gregaria* (unpublished Illumina sequencing data) with reciprocal tBLASTn (NCBI). The deduced amino acid sequences, determined by *in silico* translation using Prosite (ExPASy, ETH Zurich), were used to predict the presence of protein domains (Pfam, Sanger institute) and of a signal peptide sequence (SignalP 4.1, CBS). To validate the transcript sequence information, the cDNA fragments were cloned into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Life technologies). The sequences of the inserted DNA fragments were determined using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The transcript sequences for *tubu* (GenBank: HQ851397) and *glyceraldehyde phosphate dehydrogenase* (*gapdh*) (GenBank: HQ851387) were previously validated by Van Hiel et al. (2009).

2.3. Phylogenetic analysis

Nucleotide sequence information for prokaryotic and eukaryotic non-specific nuclease transcripts were retrieved from Genbank (NCBI), by using the *S. gregaria dsRNase* and the *C. elegans endoG* transcript sequences, and *in silico* translated into the corresponding amino acid sequence (Prosite, ExPASy). The identity of these fragments was confirmed by reciprocal tBLASTn (NCBI) and by verifying the presence of an NN-domain (Pfam, Sanger institute). Next, the amino acid sequences of the NN-domains were compared with T-coffee alignment software using the BLOSUM matrix (EMBL-EBI). The region with highest identity of approximately 100 amino acids in length was selected using Jalview sofware. The latter was used for the construction of a phylogenetic tree using the maximum likelihood method with 100 bootstraps (PhyML). Different substitution models were assessed; including models based on the LG, Dayhoff, Jones-Taylor-Thornton (JTT), Blosum62 and HIVb matrices. In addition, we assessed the Muscle alignment software (MEGA5.1). In all cases, phylogenetic trees with similar structures were obtained.

2.4. Synthesis of dsRNA

Double stranded RNAs for *dsRNase1* (576 bp), *dsRNase2* (346 bp), *dsRNase3* (496 bp), *dsRNase4* (646 bp), *tubu* (545 bp), *gapdh* (447 bp) and *green fluorescent protein* (*gfp*, 589 bp) were synthesized using the MEGAscript RNAi kit (Ambion). A DNA template flanked by two T7 promoter sequences was synthesized for production of *dsRNase1, dsRNase2, dsRNase3*, *dsRNase4, tubu* and *gapdh*. Therefore, a PCR reaction was performed with cDNA of adult *S. gregaria* midgut tissue, gene specific primers containing a T7 promoter sequence at the 5′ end (Sigma–Aldrich co.), and REDTaq mix (Sigma–Aldrich co.) as a source of DNA Taq polymerase, dNTPs and PCR buffer. All PCR primers are displayed in supplementary data (Fig. S1). The amplification products were subsequently analysed by 1% agarose gel electrophoresis and then visualized with the ProXima 2500 (Isogen Life Science) under UV-light. Moreover, the template sequences were validated by first cloning the fragments into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Life technologies) and subsequently sequencing the inserted DNA fragments by using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

The PCR product was used directly as template for production of dsRNA. Synthesis of *gfp* dsRNA was performed using a TOPO 4.1 sequencing vector (Life Technologies Co.) containing a *gfp* transcript sequence as DNA template. Since only one T7 promoter site is present in this vector, the fragment was cloned both in the sense and antisense direction. RNA was then synthesized by the T7 Enzyme Mix of the MEGAscript RNAi kit (Ambion). Both *gfp* RNA strands were first synthesized independently before being mixed to anneal, while transcripts made from a single template with opposing T7 promoters were hybridized during the transcription reaction. After the production of dsRNA, the remaining DNA and ssRNA was removed by nuclease treatment, and proteins and mono/oligonucleotides were removed by solid phase adsorption purification, according to the manufacturer's specifications (Ambion). The dsRNA concentration was determined by means of a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.), and the integrity of the dsRNA was assessed by gel electrophoresis using a 1% agarose gel. The dsRNA was stored at −20 °C until further use.

2.5. Injection and feeding of dsRNA

Locusts were (i) intra-abdominally injected, (ii) orally injected or (iii) fed with dsRNA-containing medium. *S. gregaria* (*Sg*-)Ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) was used to dilute the dsRNA to the desired concentration.

(i) Adult locusts were each intra-abdominally injected with 150 ng of dsRNA in a total volume of 10 μl. (ii) Adult desert locusts were also orally injected with 5 μl dsRNA-solution (1 or 5 μg of dsRNA). Therefore, the locusts were first starved for one day and, immediately after the oral dsRNA-injection, they were allowed to feed by supplying them with cabbage leaves. (iii) In addition, an artificial medium [2.4% wesson salt, 0.5% linoleic acid, 0.6% cholesterol, 0.3% ascorbic acid, 0.2% Vanderzant vitamin cocktail (Sigma–Aldrich co.), 54% cellulose, 14% sucrose, 14% dextrin and 14% protein (3:1:1 casein, peptone and albumin)] was used to feed dsRNA to the locusts. To solidify the medium, agarose was added to