Identification and Characterization of Pathogen-Response Genes (*repat*) in *Spodoptera frugiperda* (Lepidoptera: Noctuidae)*

Vilmar MACHADO, Jose SERRANO and Jose GALIÁN

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The fall armyworm (*Spodoptera frugiperda*, Noctuidae, Lepidoptera) is one of the most important crop pests in the Americas, causing significant damage to maize, rice and sorghum. The mechanisms that determine its defences against pathogens are particularly relevant for the development of management and control strategies. We used an *in silico* approach to identify and characterize pathogen response genes (*repat*) present in different tissue libraries of *S. frugiperda*. The analyses revealed complete cDNA for nine *repat* genes; of these, *repat15* and *repat39* were found in libraries from a specific tissue – the midgut of larvae fed with xenobiotic substances. High expression levels of some genes were found in different libraries: 39 hits in *repat30* in challenged hemocytes, 16 hits in *repat31* in fat body, 10 hits in *repat32* in fat body and 10 in challenged hemocytes, and 10 hits in *repat38* in midgut of non-treated larvae and midgut of larvae fed with natural and xenobiotic substances. The genes corresponded to two ontology categories, stress response and immune response, and their phylogenetic relationships, nucleotide similarity, number of amino acid residues and molecular weights agree with what has been described for *repat* genes. It is noteworthy that proteins encoded by the *repat* genes of *S. frugiperda* have important defence functions in other tissues beyond midgut and that their functional categories are likely diverse, as they are related to cell envelope structure, energy metabolism, transport and binding.

Key words: REPAT proteins, expressed sequences tag (EST), protein functions, immune defence, *in silico* analysis.

Vilmar MACHADO, Jose SERRANO and Jose GALIÁN, Department of Zoology and Physical Anthropology, Faculty of Veterinary, Murcia University, Campus Mare Nostrum, 30100, Murcia (Spain)
E-mail: vilmar.machado@um.es
jserrano@um.es
jgalian@um.es

The digestive tract is considered to be the main site of entrance of pathogens and toxic compounds during larval development of insects. A number of studies have shown the existence of several immune mechanisms that decrease the damage caused by pathogen infection, such as activation of detoxification and damage-repair systems and synthesis of antimicrobial proteins (LEMAITRE & HOFFMANN 2007; VILCINSKAS 2010; ZHANG et al. 2014). Thus, analysis of the midgut expression pattern of genes is important for understanding the defence mechanisms of insects against natural pathogens as well as for investigating new targets to be used in biological control strategies (GUO et al. 2009; BRAVO et al. 2011; MACEDO & FREIRE 2011; XIA et al. 2013).

The REPAT family is a group of proteins described during the study of the transcriptional response of the midgut in the armyworm *Spodoptera exigua* after infection with *Bacillus thuringiensis* (HERRERO et al. 2007; HERNÁNDEZ-RODRÍGUEZ et al. 2009). These proteins may be important for mechanisms of defence against microorganisms in this species, which are overexpressed in response to infection. Furthermore, some of these proteins were shown to be highly expressed in populations resistant to *B. thuringiensis* toxin (HERNÁNDEZ-MARTÍNEZ et al. 2010; NAVARRO-CERRILLO et al. 2012). At present, 46 members of the REPAT family have been

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identified in *Spodoptera exigua* and *S. littoralis* (Navarro-Cerrillo et al. 2013).

The fall armyworm *Spodoptera frugiperda* is a lepidopteran species that has a variety of host plants (many are important crop species) and causes economic damage due to substantial losses in agricultural production in many countries of the Americas. Currently, it is considered a major crop pest by American and European agronomic agencies (Legéat et al. 2014). Therefore, a number of studies have aimed to develop effective control methods for this pest species, and there is also considerable interest in understanding the mechanisms that allow this species to exploit different plant species, by having lineages adapted to various host plants that respond differently to chemical and biological control agents (Nagoshi et al. 2007; Machado et al. 2008; Groot et al. 2008; Barros et al. 2010; Velasquez-Velez et al. 2011; 2014; Devappa et al. 2012; Carvalho et al. 2013; Jakka et al. 2014).

The construction of cDNA libraries is a useful way to identify genes with differential expression in different tissues or different developmental stages (Duan et al. 2013; Lenz et al. 2013; Robert et al. 2013; Kim et al. 2014; Nanoth-Velllichirammal et al. 2011; Benoit et al. 2014), generating a body of knowledge important for applied biology, particularly for pest control strategies.

Bioinformatics based analyses (*in silico* transcriptome mining) has become a relevant tool to provide means for the identification and characterization of proteins of interest. The information obtained by *in silico* investigations is badly needed particularly when there is a lack of *in vivo* information, as it provides the basis for approaches based on experimental work (Christie et al. 2013; 2014; Nesbit & Christie 2014).

The aim of this work was to identify pathogen response genes (*repat*) of *Spodoptera frugiperda* through *in silico* comparative expressed sequence tag (ESTs) analyses from one assembled reference library and five unassembled tissue specific libraries (treated and non-treated) and to characterize their gene functions and subcellular localizations using bioinformatic tools. In particular, we aimed to: 1) identify and characterize *repat* genes present in five *S. frugiperda* EST libraries made from different treated and non-treated tissues; 2) analyse the distribution of these genes in the five libraries; 3) explore the possibility of inferring information on the expression of these genes in different tissues based on the frequency of the EST for each gene. It is expected that the resulting information will be relevant for studies involved in the understanding of defence mechanisms of *S. frugiperda*, and the evolution of strains resistant to microbial infections used in biological control strategies of this pest.

**Material and Methods**

Expressed sequences tag (ESTs)

The identification of *repat* genes was based on the analysis of SF_TR2012b assembled sequences downloaded from the Lipidodb database (http://www6.inra.fr/lepidodb/Private), a centralized bioinformatics resource to facilitate the comparative genomics of two major lepidopteran pests, the noctuid moths *Helicoverpa armigera* and *Spodoptera frugiperda*. Additionally, five EST unassembled libraries from the *Spodoptera* database (SPODABASE – http://bioweb.ensam.inra.fr/spodbase/) were downloaded as well. These five EST libraries were: SF1F (fat body), SF1H (hemocytes), SF2H (immune challenged hemocytes (larvae infected with polydnaviruses), Provost et al. 2011), SF1M (midgut), and SF2M (larvae fed on artificial diet supplemented with various natural products and xenobiotics (Negre et al. 2006). These five libraries were assembled with SOAP2novo with the default settings available in the Japan Supercomputer DDBJ website (Nagasaki et al., 2013), and were annotated with Blast2go software (Conesa et al. 2005) to detect the presence of *repat* genes.

Sequence analysis

The identification of *repat* genes in *Spodoptera frugiperda* was based on homology analysis using Blast searches against the Sf_TR2012b EST library, with an e-value <10^-5, using the BLOSUM62 matrix and other default parameters against *repat* genes described for other *Spodoptera* species. All sequences with significant Blast results (e-value <10^-5) were analysed by ORFFinder to detect the presence of complete open reading frames (ORF). The complete ORFs were blasted to the NCBI data set to confirm homology. We selected the sequences with the best e-value and high similarity with *repat* gene sequences (sequences in supplementary data).

To obtain more information on functional and gene ontology categories, the 10 REPAT proteins identified in *S. frugiperda* (SF_TR2012b) and their homologue sequences from the database from the congeneric species *S. exigua* and *S. littoralis* were analysed using the ProtFun server (http://www.cbs.dtu.dk/services/ProtFun/), an efficient tool to validate gene function (Jensen et al. 2002; Sommer et al. 2004). Predictions made by ProtFun rely on criteria derived from the analysis of known features in already annotated proteins, including post-transcriptional modifications (specific protein signals) and chemical and physical properties. The presence/absence of signal peptide was predicted by using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The potential subcellular localization of proteins was
predicted using ProtComp Version 9.0 (http://www.softberry.com).

The frequency of each repat EST in each of the five unassembled tissue libraries mentioned above was used as an indicator of their expression level by applying the statistical test suggested by ROMUALDI et al. (2001). The Blast search was done with 1e-30 and the results were carefully revised to eliminate sequences with two or more matches. The best scores were selected to avoid assigning any given EST to more than one protein. This approach was used to evaluate the differences in gene expression in each library. Only hits with more than 95% similarity were further considered.

Phylogenetic analysis

Alignment of the amino acid sequences was performed using the default parameters of MUSCLE (EDGAR 2004). Phylogenetic relationships were estimated by maximum likelihood analysis software PHYML v3.0 (GUINDON et al. 2010) with 1000 bootstrap replicates. This analysis used 13 REPAT proteins of S. exigua and 3 of S. littoralis (in this species the number of described REPAT proteins is lower) that showed the highest homology to S. frugiperda proteins.

Results

The blast analysis showed the presence of nine REPAT proteins in the cDNA library of S. frugiperda (Table 1). Genes were distributed in different tissues; four repat members were found in midgut from larvae treated with natural and xenobiotic substances, four in midgut of non-treated larvae, two in hemocytes of non-treated larvae, four in hemocytes of larvae infected with polydnaviruses, and three in the fat body of larvae infected with polydnaviruses.

The similarity with homologous repat genes found in S. exigua ranged from 57% (repat 18) to 98% (repat 38). In five genes, the similarity was higher than 85%. The lowest similarity values were found in repat 13 and repat 15. The number of amino acid residues present in S. frugiperda REPAT proteins were exactly the same as those found in homologous in five cases (REPA T30, REPAT31, REPAT32, REPAT33 and REPAT39), with differences of one residue in REPAT13, REPAT15 and REPAT38. Larger differences in the number of residues were found in REPAT18, with four residues more than its homologue in S. exigua. Only nine of these genes exhibited complete ORFs. Incomplete ORFs were found in repat 1, repat 14 and repat 27 of the S. frugiperda library.

The relationships of the REPAT proteins described based on ML methods are shown in Figure 1. The phylogenetic analysis included 12 REPAT proteins from S. exigua. In this analysis the distribution of REPAT proteins in S. frugiperda showed a similar pattern to that obtained by NAVARROCERRILLO et al. (2013), and consisted of the fol-

<table>
<thead>
<tr>
<th>Gene</th>
<th>cDNA pb</th>
<th>Amino acids number S. frugiperda</th>
<th>Amino acids number Homologous</th>
<th>Peptide signal</th>
<th>E-value</th>
<th>Similarity</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>repat13</td>
<td>408</td>
<td>135</td>
<td>134</td>
<td>Yes</td>
<td>1e^-53</td>
<td>66%</td>
<td>AFH57133.1 S. exigua</td>
</tr>
<tr>
<td>repat15</td>
<td>474</td>
<td>157</td>
<td>156</td>
<td>yes</td>
<td>9e^-65</td>
<td>63%</td>
<td>AFH57135.1 S. exigua</td>
</tr>
<tr>
<td>repat18</td>
<td>387</td>
<td>128</td>
<td>132</td>
<td>yes</td>
<td>1e^-44</td>
<td>57%</td>
<td>AFH57138.1 S. exigua</td>
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<tr>
<td>repat30</td>
<td>360</td>
<td>119</td>
<td>119</td>
<td>yes</td>
<td>8e^-64</td>
<td>94%</td>
<td>AFH57169.1 S. littoralis</td>
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<tr>
<td>repat31</td>
<td>333</td>
<td>110</td>
<td>110</td>
<td>yes</td>
<td>1e^-59</td>
<td>92%</td>
<td>AFH57151.1 S. exigua</td>
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<tr>
<td>repat32</td>
<td>333</td>
<td>110</td>
<td>110</td>
<td>yes</td>
<td>1e^-72</td>
<td>98%</td>
<td>AFH57171.1 S. littoralis</td>
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<tr>
<td>repat33</td>
<td>300</td>
<td>109</td>
<td>109</td>
<td>yes</td>
<td>6e^-33</td>
<td>92%</td>
<td>AFH57153.1 S. exigua</td>
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<tr>
<td>repat38</td>
<td>369</td>
<td>122</td>
<td>121</td>
<td>yes</td>
<td>1e^-48</td>
<td>92%</td>
<td>AFH57158.1 S. exigua</td>
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<tr>
<td>repat39</td>
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<td>151</td>
<td>151</td>
<td>yes</td>
<td>2e^-64</td>
<td>85%</td>
<td>AFH57167.1 S. littoralis</td>
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</tbody>
</table>
lowing groups: I (REPAT 13, 15, 18); III (REPAT 30, 31, 31, 33) and IV (REPAT39). An exception to this pattern occurred with REPAT38 as it did not cluster together with that of *S. exigua* in group V, but both are in distinct groups equally distant from the cluster consisting of groups I, III and IV of NAVARRO-CERRILLO et al. (2013). However these two proteins have the same function according to evaluation in Protfun.

Fours proteins were detected just in one specific library and five were present in two or more tissue libraries (Table 2). The frequency of the EST in each library showed high expression of some genes in different libraries. According to the

Table 2

Functional annotation and EST presence of *Spodoptera frugiperda* REPAT proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Functional category</th>
<th>Enzyme</th>
<th>Gene Ontology category (GO)</th>
<th>GO S. Exigua</th>
<th>Migdut Xe</th>
<th>Midgut</th>
<th>Hemoocytes</th>
<th>Challenged Hemoocytes</th>
<th>Fat body</th>
</tr>
</thead>
<tbody>
<tr>
<td>REPAT13</td>
<td>Cell envelope</td>
<td>Y</td>
<td>Stress response</td>
<td>Stress</td>
<td>X(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT15</td>
<td>Cell envelope</td>
<td>N</td>
<td>Stress response</td>
<td>Stress</td>
<td>X(6)</td>
<td>X(1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT18</td>
<td>Cell envelope</td>
<td>Y</td>
<td>Immune response</td>
<td>Immune</td>
<td>X(4)</td>
<td>X(1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT30</td>
<td>Cell envelope</td>
<td>N</td>
<td>Immune response</td>
<td>Immune</td>
<td>X(6)</td>
<td>X(39)</td>
<td>X(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT31</td>
<td>Cell envelope</td>
<td>N</td>
<td>Immune response</td>
<td>Immune</td>
<td>X(4)</td>
<td></td>
<td>X(15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT32</td>
<td>Cell envelope</td>
<td>Y</td>
<td>Immune response</td>
<td>Immune</td>
<td>X(2)</td>
<td>X(10)</td>
<td>X(10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT33</td>
<td>Cell envelope</td>
<td>N</td>
<td>Stress response</td>
<td>Stress</td>
<td>X(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT38</td>
<td>Cell envelope</td>
<td>Y</td>
<td>Immune response</td>
<td>Immune</td>
<td>X(20)</td>
<td>X(17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REPAT39</td>
<td>Cell envelope</td>
<td>N</td>
<td>Stress response</td>
<td>Stress</td>
<td>X(14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

( ) Hit number for the genes in the libraries in brackets.
Midgut Xe= Midgut Xenobiotic
number of ESTs found in each library, it was inferred that some genes were highly expressed: 39 hits in repat30 in challenged hemocytes, 15 hits in repat31 in fat body, 10 hits in repat32 in the fat body and 10 in challenged hemocytes, 16 in repat38 in midgut and 10 in midgut of larvae fed with natural and xenobiotic substances, and 14 in repat39 in midgut of non-treated larvae.

The Protfun analysis showed that all nine proteins have the same functional category (cell envelope) and can be associated with two ontology categories, namely, stress response (REPAT13, REPAT15, REPAT33 and REPAT39) and immune response (REPAT14, REPAT18, REPAT30, REPAT31, REPAT32 and REPAT33). The stress response category was found in libraries from midgut of larvae treated with xenobiotic substances, midgut from non-treated larva and challenged hemocytes. The same functional and ontological categories were found for homologous REPAT proteins from S. exigua. All were classified as secretory proteins by ProtComp.

The physiochemical properties of REPAT proteins of S. frugiperda obtained by ProtFun are shown in Table 3. The molecular weight is less than 20 Daltons as expected for these proteins. Theoretical isoelectric points indicate the presence of three acids (REPAT 13, 18 and 27) and seven basic proteins (15, 30, 31, 32, 33, 38, and 39). The instability index showed that most of these proteins are stable except for REPAT 13 and 18. Almost all values obtained in computational physiochemical analyses are similar to those found in homologous REPAT from S. exigua. The exception was REPAT 13, classified as stable and REPAT 39 classified as unstable in S. exigua, while in our study these proteins were classified as the opposite.

### Discussion

The genes described in this study can be considered as members of the REPAT family based upon several features, such as phylogenetic relationship, nucleotide similarity, number of amino acid residues and molecular weight, as described by NAVARRO-CERRILLO et al. (2013). Additionally, these proteins showed the same functional and ontological categories found in their homologous described in S. exigua, i.e., immune and stress responses.

An important question in the study of the REPAT proteins is concerned with the tissue where the main expression and specific function occur. Previous studies have shown the importance of REPAT proteins in immune response during midgut infection by pathogens (HERRERO et al. 2007; HERNÁNDEZ-RODRÍGUEZ et al. 2012; NAVARRO-CERRILLO et al. 2012, 2013). However, we have detected five REPAT proteins from EST libraries of different tissues, showing different expression patterns based on EST frequency. The presence of these proteins in tissues other than midgut was mentioned previously (NAVARRO-CERRILLO et al. 2013), but those authors concluded that the main tissue for REPAT expression is midgut, as these proteins were not found in the analysis of fat body and hemocytes of S. exigua infected with Bacillus thuringiensis toxins and baculoviruses (HERRERO et al. 2007). The presence of some specific REPAT proteins in fat body and hemocytes indicates a more widespread action of these proteins in S. frugiperda, which are likely involved in various processes, such as stress response and immune function, as indicated by ProtFun analysis. Another indication of the functional relevance of REPAT proteins in other tis-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino acid residues</th>
<th>Molecular weight (kDa)</th>
<th>Theoretical pl</th>
<th>Negatively charged residues (Asp + Glu)</th>
<th>Positively charged residues (Arg + Lys)</th>
<th>Extinction coefficients</th>
<th>Instability index</th>
<th>Aliphatic index</th>
<th>Grand average of hydro-pathicity</th>
</tr>
</thead>
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<tr>
<td>repat13</td>
<td>135</td>
<td>14719.7</td>
<td>4.70</td>
<td>14</td>
<td>9</td>
<td>16960</td>
<td>40.85</td>
<td>104.67</td>
<td>0.173</td>
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<tr>
<td>repat15</td>
<td>157</td>
<td>17061.4</td>
<td>7.86</td>
<td>11</td>
<td>12</td>
<td>13410</td>
<td>27.57</td>
<td>99.94</td>
<td>0.086</td>
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<tr>
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<td>128</td>
<td>14243.3</td>
<td>4.79</td>
<td>11</td>
<td>8</td>
<td>19940</td>
<td>50.59</td>
<td>103.52</td>
<td>0.267</td>
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<tr>
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<td>119</td>
<td>13175.5</td>
<td>9.38</td>
<td>9</td>
<td>14</td>
<td>13410</td>
<td>12.99</td>
<td>93.36</td>
<td>0.001</td>
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<tr>
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<td>12067.0</td>
<td>9.23</td>
<td>8</td>
<td>12</td>
<td>13075</td>
<td>31.82</td>
<td>102.73</td>
<td>0.233</td>
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<tr>
<td>repat32</td>
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<td>12230.3</td>
<td>8.98</td>
<td>10</td>
<td>13</td>
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<td>34.64</td>
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<tr>
<td>repat33</td>
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<td>9.55</td>
<td>5</td>
<td>11</td>
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<td>112.66</td>
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<tr>
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<td>122</td>
<td>13788.6</td>
<td>9.62</td>
<td>6</td>
<td>12</td>
<td>28880</td>
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<td>88.85</td>
<td>-0.231</td>
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<tr>
<td>repat39</td>
<td>151</td>
<td>16291.5</td>
<td>9.68</td>
<td>12</td>
<td>15</td>
<td>7450</td>
<td>36.99</td>
<td>111.79</td>
<td>0.120</td>
</tr>
</tbody>
</table>
sues is the fact that REPAT30 and 31 are notably expressed in challenged hemocytes and fat body. Furthermore, ProtFun analysis of complete REPAT protein sequences described in Spodoptera showed that REPAT 5, 14, 25 and 26 proteins are included in the transport and binding functional categories, and that REPAT44 is involved in energy metabolism, in addition to the cell envelope category already indicated for most of them. Moreover, gene ontology category analysis showed that REPAT42 has a structural function besides immune and stress responses.

Analysis of the library of hemocytes and fat body of larvae infected with polyadenoviruses showed a high expression level of genes related to immunity, detoxification and maintenance of cell structure (BRAVO et al. 2011). NAVARRO-CERRILLO et al. (2012) put forward the possibility of the existence of REPAT proteins with different ontological functions, based on differences in their subcellular localization, expression pattern and interactions. To these authors the up-regulation of the REPAT protein might also be an answer to cellular damage and not only to bacterial infection.

The frequency of ESTs in the analysed libraries may indicate high expression levels of REPAT30 and 31, both of which are involved in immune response in hemocytes of infected larvae, as expected from the main function attributed to REPAT proteins. Moreover, REPAT39 may be associated with stress response, as it showed high expression in midgut of larvae submitted to stress conditions (i.e., fed with xenobiotic substances); this is the case of REPAT38 related to immune response that showed a high level of expression as well.

These expression patterns point to the existence of differences in expression rates of REPAT proteins in different tissues under different conditions. However, the interpretation of these results is difficult at the moment, as a library from midgut tissues is unavailable. Additionally, our analyses found the presence of only nine REPAT genes in the EST libraries of S. frugiperda. These results might be due to the fact that most REPAT proteins are over expressed in midgut, but only as a response to infection (HERRERO et al. 2007; HERNÁNDEZ-RODRÍGUEZ et al. 2012; NAVARRO-CERRILLO et al. 2013).

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