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Functional analysis of an immune gene of *Spodoptera littoralis* by RNAi

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ABSTRACT

Insect immune defences rely on cellular and humoral responses targeting both microbial pathogens and metazoan parasites. Accumulating evidence indicates functional cross-talk between these two branches of insect immunity, but the underlying molecular mechanisms are still largely unknown. We recently described, in the tobacco budworm *Heliothis virescens*, the presence of amyloid fibers associated with melanogenesis in immune capsules formed by hemocytes, and identified a protein (P102) involved in their assembly. Non-self objects coated by antibodies directed against this protein escaped hemocyte encapsulation, suggesting that P102 might coordinate humoral and cellular defence responses at the surface of foreign invaders. Here we report the identification of a cDNA coding for a protein highly similar to P102 in a related Lepidoptera species, *Spodoptera littoralis*. Its transcript was abundant in the hemocytes and the protein accumulated in large cytoplasmic compartments, closely resembling the localization pattern of P102 in *H. virescens*. RNAi-mediated gene silencing provided direct evidence for the role played by this protein in the immune response. Oral delivery of dsRNA molecules directed against the gene strongly suppressed the encapsulation and melanization response, while hemocoelic injections did not result in evident phenotypic alterations. Shortly after their administration, dsRNA molecules were found in midgut cells, en route to the hemocytes where the target gene was significantly down-regulated. Taken together, our data demonstrate that P102 is a functionally conserved protein with a key role in insect immunity. Moreover, the ability to target this gene by dsRNA oral delivery may be exploited to develop novel technologies of pest control, based on immunosuppression as a strategy for enhancing the impact of natural antagonists.

1. Introduction

Insect cuticle is an effective physical barrier preventing the entrance into the haemocoel of a number of foreign invaders, which is complemented by a powerful innate immune system, active against pathogens and parasites. The recognition of non-self intruders immediately activates a number of defence reactions, mediated by circulating molecules and immune cells, which are commonly referred to as humoral and cellular immunity, respectively (Lemaitre and Hoffmann, 2007; Kounatidis and Ligoxygakis, 2012). However, these two virtual arms of the immune response are basically different components of an unique machinery, since they are inextricably linked and reciprocally modulate their function through complex interactions, only partially understood (Lemaitre and Hoffmann, 2007; Cerenius et al., 2008). Insect pathogens and parasitoids are able to successfully overcome these immune barriers by using virulence factors, which often disrupt the melanization response (Pennacchio and Strand, 2006; Eleftherianos et al., 2007; Beck and Strand, 2007; Lu et al., 2008; Colinet et al., 2009; Strand, 2012). However, how this effect is associated with suppression of encapsulation (i.e. the formation of a cellular multilayer enclosing non-self elements) is not well defined, and there is also some unresolved controversy on the general assumption that melanogenesis is an absolute requirement for killing parasites and pathogens (Cerenius et al., 2008; Nappi and Christensen, 2005).

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We recently discovered that an insect immune gene, highly expressed in the hemocytes of *Heliothis virescens* and denoted as *102*, encodes a protein (P102) which generates around non-self objects a layer of amyloid fibers associated with melanin (Falabella et al., 2012). The active role of amyloids in insect melanogenesis appears to closely mimic the strategy used by mammals to cope with the intrinsic toxicity of the intermediate metabolites in the melanin biosynthetic pathway. Indeed, the conserved structural characteristics of the amyloid molecular scaffold allow tight binding of cytotoxic melanin precursors, hastening their polymerization and preventing the lethal diffusion of these molecules outside of the confined compartment where melanogenesis takes place (Fowler et al., 2006, 2007). Moreover, we also provided indirect evidence that the *102* gene might modulate the cellular immune response, as G Sepharose beads injected into the larval hemocoel were poorly encapsulated when coated with anti-P102 antibodies (Falabella et al., 2012). Collectively, the reported experimental data indicate a central role of this gene in the immune response, but how this is exerted and if a conserved mechanism exists in other lepidopteran species still remain open questions.

Here we address these issues, using a related moth species, *Spodoptera littoralis*, a polyphagous pest of remarkable economic importance, occurring in many regions of Africa, Asia and Europe (http://www.eppo.int/QUARANTINE/insects/Spodoptera_litura/PRODLI_ds.pdf). We obtained and characterized a full-length cDNA encoding a protein with high sequence similarity to P102. To characterize this protein from a functional point of view and to establish if and how it is involved in the modulation of immune response we silenced its coding gene by RNAi. This approach allowed us to gather direct evidence on the role of this gene in insect immune response.

The use of RNAi in Lepidoptera has been widely pursued, but with more difficulties compared to other insect orders (Terenius et al., 2011). The causes of these problems are often unknown, and the registered failures could be due to a combination of different factors, such as, for example, the life stage and the amount of dsRNA used, the gene itself and its predominant site of expression (Yu et al., 2013). However, a fairly good number of studies on Lepidoptera are encouraging, in particular those reporting effective RNAi induced by oral delivery of dsRNA (Turner et al., 2006; Mao et al., 2007; Surakasi et al., 2011). With this in mind, we first defined an effective protocol to orally deliver controlled amounts of dsRNA to the experimental larvae, able to induce RNAi in the tissue where the target gene is predominantly expressed. Then, we studied the phenotypic alterations associated with gene knockdown, by developing a simple bioassay to score the immune response against non-self objects injected into the hemocoel. The obtained results shed light on the functional role played by the immune gene studied and set the stage for future development of novel pest control technologies, based on RNAi strategies.

2. Materials and methods

2.1. Insect material

S. littoralis larvae were individually reared on artificial diet (41.4 g/L wheat germ, 59.2 g/L brewer's yeast, 165 g/L corn meal, 5.9 g/L ascorbic acid, 1.53 g/L benzoic acid, 1.8 g/L methyl 4-hydroxybenzoate and 29.6 g/L agar), at 25 ± 1 °C and 70 ± 5% RH, with 16:8 h light-dark period.

2.2. Hemocyte collection

Larval hemolymph was collected from a cut of the abdominal proleg into ice-cold MEAD anti-coagulant buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid, pH 4.5). Hemocytes were separated from plasma by 5 min centrifugation at 500×g, at 4 °C.

2.3. Identification of 102 Sl cDNA

A partial *102 Sl* cDNA (Accession Number FQ016824.1) was identified by BLAST analyses (Altschul et al., 1997; Gish and States, 1993) in a public database of EST sequences from *S. littoralis* male antenna, using as query the sequence of the *H. virescens 102* cDNA (Accession Number FR751090). The predicted 102 *Sl* protein (P102 *Sl*) and P102 sequences (Accession Number CBY85302.1) were aligned using the Clustal W algorithm.

The full-length *102 Sl* cDNA sequence was obtained by using the 3′ and 5′ RACE System for Rapid Amplification of cDNA Ends (Life Technologies, Carlsbad, CA, USA), following manufacturer's instruction. *102 Sl* gene specific primers used for nested PCR reactions in the 3′ RACE experiment were *3′GSP1 F* (TACATCCAAG TAAATTTGCAAGGC) and *3′GSP2 F* (CGCTACAACAACGTCAACTT). In the 5′ RACE experiment, *5′GSP1 R* (TGAGTCCGATTGAACA) was used as gene specific primer for reverse transcriptase while *5′GSP2 R* (CTCGTAGCTCTTCTTCTTGGCATA) and *5′GSP3 R* (ATCGACGACACCG CCTACTA) were used for nested PCR reactions. RACE products were purified from low-melting agarose and sequenced directly by using GSPs as sequencing primers.

2.4. RNA extraction

Total RNA was extracted from *S. littoralis* fifth instar larvae and from hemocytes, integument, fat body and gut, using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), according to manufacturer's instructions.

2.5. dsRNA synthesis

Total RNA purified from hemocytes of *S. littoralis* sixth instar larvae was retro-transcribed with the RETRO script® Kit (Life technologies, Carlsbad, CA, USA) and a 580 bp long *102 Sl* cDNA fragment was obtained by PCR using the *3′GSP1 F* primer (see above) in combination with the *102Sl Rev* primer (GGCCCAGAACATTCTC ACCTC). This cDNA fragment was used as template for a nested-PCR reaction, performed with primers containing at their 5′ ends the T7 polymerase promoter sequence (*T7-102Sl F*: TAATACGACTC ACTATAGGGAGAACCTCCTGAGCGTGCCTGA; *T7-102Sl R*: TAATAC GACTCACTATAGGGAGGGAGTGCTGCTTCAGAATCAT). The resulting PCR product served in turn as template to synthesize dsRNAs (469 bp long), using the Ambion MEGAscript® RNAi Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. dsRNA preparations were quantified by measuring their absorbance at 260 nm with a Biophotometer (Eppendorf) and purity was evaluated by assessing 260/280 nm absorbance ratios. Products were run on 1% agarose gels to cofirm their integrity.

2.6. Administration of dsRNA to S. littoralis larvae

S. littoralis fourth instar larvae (first day) were anaesthetized by immersion in sterile water and 1 μl of *102 Sl* dsRNA or GFP dsRNA (control) solution was poured into the lumen of the foregut by means of a Hamilton Microliter syringe (1701RNR 10 μl, gauge 26s, length 55 mm, needle 3). dsRNA treatments consisted of two oral administrations per day (every 12 h), for 3 days (from fourth to fifth instar). dsRNA solutions at three different concentrations were used: 4.5, 45 and 450 ng/μl. 12 h after the last dsRNA administration, treated larvae were processed for quantitative RT-PCR analysis or injected with chromatografic beads. Immune-challenged larvae were subjected to the encapsulation assay (see