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RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*

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Abstract

Bacterial pathogens either hide from or overcome the immune response of their hosts. Here we show that two different species of insect pathogenic bacteria, *Photorhabdus luminescens* TT01 and *Photorhabdus asymbiotica* ATCC43949, were both recognized by the immune system of their host *Manduca sexta*, as indicated by a rapid increase in the levels of mRNAs encoding three different inducible microbial recognition proteins, Hemolin, Immulectin-2 and peptidoglycan recognition protein. RNA interference (RNAi)-mediated inhibition of expression (''knock-down'') of each of these genes at the level of both mRNA and protein was achieved through injection of double-stranded RNA (dsRNA). Knock-down of any one of these genes markedly decreased the ability of the insects to withstand infection when exposed to either species of *Photorhabdus*, as measured by the rate at which infected insects died. RNAi against Immulectin-2 caused the greatest reduction in host resistance to infection. The decreased resistance to infection was associated with reduced hemolymph phenoloxidase activity. These results show not only that *Photorhabdus* is recognized by the *Manduca sexta* immune system but also that the insect's immune system plays an active, but ultimately ineffective, role in countering infection.

Keywords: RNAi; Hemolin; Immulectin-2; Peptidoglycan recognition protein; *Photorhabdus*; *Manduca sexta*

1. Introduction

Pathogens either hide from the host immune system in specific immune protected sites or modulate the host immune response to ensure their survival [1–4]. In the case of the insect pathogen *Photorhabdus*, bacteria are introduced into the host insect via a specific nematode vector [5,6]. Once the infective juvenile nematode has penetrated the cuticle of the insect host, the bacteria are regurgitated from the nematode gut directly into the open blood system (hemocoel) of the insect [6,7]. At this stage they are potentially subject to detection by the host immune system, resulting in transcription of immune-related genes, triggering the production of

Abbreviations: RNAi, RNA interference; dsRNA, double-stranded RNA; IML-2, Immulectin-2; PGRP, peptidoglycan recognition protein; PO, phenoloxidase; proPO, pro-phenoloxidase

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microbial pattern recognition proteins and antibacterial peptides from the fat body. Once recognized, the bacteria are also subject to phagocytosis and nodulation by insect phagocytes (hemocytes) [6]. However, during the course of normal infection *Photorhabdus* multiplies within the insect, producing a range of insecticidal toxins that quickly kill its host [6]. The vector nematodes then reproduce, feeding off the growing bacteria they have released, until a new generation of infective juveniles is produced which re-uptake the bacteria and exit the cadaver to find further insect hosts [6]. Although the net result of this interaction is always assumed to be insect death, we were interested to establish if *Photorhabdus* bacteria are recognized by the immune system of its lepidopteran host *Manduca sexta* and, if so, whether the immune system plays any role in controlling the growing bacterial population within the insect.

To investigate if *Photorhabdus* is recognized by the host immune system we focused on three key recognition proteins: Hemolin, Immulectin-2 (IML-2) and peptidoglycan recognition protein (PGRP). Hemolin is a pattern recognition molecule exclusive to Lepidoptera and is involved in both humoral and cellular defence [8,9]. Hemolin can bind both hemocytes and bacteria and may be involved in opsonization or in trapping bacteria in the nodules formed by hemocytes [9]. Silencing of Hemolin in the silkmoth *Antheraea pernyi* has also been shown to affect the progress of virus infection [10]. Further, RNA interference (RNAi)-mediated knock-down of Hemolin in *Hyalophora cecropia* pupae appears to be lethal to the next generation of embryos, implying that it may also play a role in insect development [11]. IML-2 binds to serine proteases in insect plasma that activate pro-phenoloxidase (proPO) to active phenoloxidase or PO [9]. IML-2 has been shown to be important in protecting against Gram-negative bacteria and may function by localizing the PO response to the surface of invading bacteria [9]. Finally, PGRPs recognize peptidoglycan in bacterial cell walls. PGRP was first purified from silkworm hemolymph via its high affinity for peptidoglycan and its ability to trigger peptidoglycan-dependent activation of the proPO cascade [12]. Two PGRP genes, PGRP-1A and PGRP-1B, have been cloned from *Manduca sexta,* which differ in their nucleotide sequences in only very minor respects [13]. The mature proteins are identical, their amino acid sequences differing only in their leader sequences. In *Manduca sexta*, PGRP mRNA is expressed at a low level in the fat body but can be up-regulated as early as 2 h after bacterial injection [13]. It is not known whether the PGRP-1A and PGRP-1B genes are differentially regulated. All three recognition proteins, Hemolin, IML-2 and PGRP, would be expected to be important in the recognition of the Gram-negative pathogen *Photorhabdus*, if indeed the bacteria are recognized by the immune system following release from their nematode vector.

RNAi has been effectively employed to confirm the functional role of proteins involved in the insect immune response to both *Plasmodium* [14] and parasitic wasps [15]. In the latter case, depletion of the hemocyte-specific *Drosophila* protein Hemese caused an unexpected enhanced response to the wasp, suggesting that this protein may be involved in regulating the extent of the anti-wasp immune response [15]. In Lepidoptera, RNAi has been used to knock-down the expression of nicotinic acetylcholine receptors in the nervous system of *Manduca sexta* [16] and to alter eye color via knock-down of tryptophan oxidase [17]. Similarly, RNAi has also been used to study the release of sperm from moth testes [18], to suppress expression of aminopeptidase N, a midgut receptor for insecticidal δ-endotoxins from *Bacillus thuringiensis* [19], to demonstrate the importance of carotenoid binding protein in silkmoth cocoon pigmentation [20] and to show that a hemocyte-specific integrin is involved in encapsulation of polymer beads [21]. However, studies using RNAi to modulate lepidopteran immune responses to insect pathogens have so far been lacking.

Here we address two fundamental questions relating to insect infection by the pathogen *Photorhabdus*. First, following injection of *Photorhabdus* directly into the hemocoel, are the bacterial cells recognized by immune recognition proteins? In answering this question experimentally it is important to use a realistic dose of the pathogen, because the number of bacterial cells introduced into the insect host by the nematode vector is known to be small [22]. Second, if the pathogen is recognized, does the subsequent immune response play any role in altering the rate at which the infected host insect dies? We show here not only that *Photorhabdus* is recognized by the insect immune system, but also that RNAi-mediated knock-down of any of the three recognition genes tested significantly speeds insect death. In the case of IML-2 this