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Effect of host plant and immune challenge on the levels of chemosensory and odorant-binding proteins in caterpillar salivary glands

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ABSTRACT

More than half of the proteome from mandibular glands in caterpillars is represented by chemosensory proteins. Based on sequence similarity, these proteins are putative transporters of ligands to gustatory receptors in sensory organs of insects. We sought to determine whether these proteins are inducible by comparing, both qualitatively and quantitatively, the salivary (mandibular and labial) proteomes from caterpillars (*Vanessa cardui*) reared on different plants and artificial diet containing either bacteria or bacterial cell-walls. We included a treatment where the caterpillars were switched from feeding on artificial diet to plant material at some point in their development. Additionally, we evaluated the degree of overlap between the proteomes in the hemolymph-filled coelom and salivary glands of caterpillars reared on plant material. We found that the quality and quantity of the identified proteins differed clearly between hemolymph-filled coelome, labial and mandibular glands. Our results indicated that even after molting and two-day feeding on a new diet, protein production is affected by the previous food source used by the caterpillar. Candidate proteins involved in chemosensory perception by insects were detected: three chemosensory (CSPs) and two odorant-binding proteins (OBPs). Using the relative amounts of these proteins across tissues and treatments as criteria for their classification, we detected hemolymph- and mandibular gland-specific CSPs and observed that their levels were affected by caterpillar diet. Moreover, we could compare the protein and transcript levels across tissues and treatment for at least one CSP and one OBP. Therefore, we have identified specific isoforms for testing the role of CSPs and OBPs in plant and pathogen recognition. We detected catalase, immune-related protein and serine proteases and their inhibitors in high relative levels in the mandibular glands in comparison to the labial glands. These findings suggest that the mandibular glands of caterpillars may play an important role protecting the caterpillar from oxidative stress, pathogens and aiding in digestion. Contamination with hemolymph proteins during dissection of salivary glands from caterpillars may occur but it is not substantial since the proteomes from hemolymph, mandibular and labial glands were easily discriminated from each other by principal component analysis of proteomic data.

1. Introduction

Molecules detected in caterpillar saliva and other insect secretions have been found to mediate the interaction between plants and herbivores (Mithofer and Boland, 2008) by either suppressing (Weech et al., 2008) or activating plant defenses (Schmelz et al., 2009). These molecules have been defined as herbivore-

Abbreviations: ACN, acetonitrile; BGRP, beta-glucan recognition protein; CSP(s), chemosensory protein(s); EST(s), expressed sequence tag(s); HAMPs, herbivore-associated molecular patterns; LC-MS/MS, liquid chromatography tandem mass spectrometry; MRSP, methionine-rich storage protein; OBP(s), odorant-binding protein(s).

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associated molecular patterns (HAMPs). HAMPs such as glucose oxidase (Musser et al., 2005a; Tian et al., 2012) and caeliferin (sulfated fatty acid) (Schmelz et al., 2009) produce a burst of jasmonic acid in the host plant which in turn activates a defense response cascade against herbivory. Moreover, it has been observed that HAMPs in labial saliva of caterpillars could reduce the infectivity of bacterial pathogens (Musser et al., 2005b). The importance of HAMPs has drawn interest into the study of the composition of insect saliva, aiming to understand the evolutionary relationship between herbivores and their hosts (Musser, 2005).

A single type of chemosensory protein (CSP) represents about half of all soluble proteins in the salivary glands of caterpillars by proportion of total protein mass (Celorio-Mancera et al., 2012). The biological function of most CSPs in Arthropoda remains elusive regardless of their high level of conservation. CSPs seem to transport chemical cues within insect sensilla while others may be associated with completely different processes, such as organ regeneration (Pelosi et al., 2006). In addition, this type of small protein has been identified in both sensory and non-sensory tissues (Gong et al., 2007; Jacquin-Joly et al., 2001; Liu et al., 2010; Picimbon et al., 2001). Therefore, the identification of CSPs through sequence similarity barely suggests the discovery of putative proteins belonging to a multifunctional gene family of hydrophobic-ligand carriers (Pelosi et al., 2006). Nevertheless, it has been proposed that CSPs sequester or store plant-derived chemicals in the insect body as a strategy against predation (The Heliconius Genome Consortium, 2012).

Caterpillar CSPs secreted during feeding are possible factors determining acceptability or rejection of a plant. We consider that the larval stage of herbivorous insects makes the ultimate host-plant selection. Tasting is critical for the acceptability or rejection of food by insect herbivores (Chapman, 2003). Larvae can often move between hosts and locate new feeding sites (Bernklau et al., 2009; Cunningham et al., 2011; Jones and Coaker, 1977) perceiving with their sensilla in the maxillae and eipipharynx (Hansson, 1995; Schoonhoven and van Loon, 2002) chemicals in the leaf surface (Chapman and Bernays, 1989). Moreover, it has been observed that leaf and root herbivores are even able to exploit those herbivore-induced plant volatiles for host plant location (Carroll et al., 2008; Robert et al., 2012).

It is necessary to determine whether the function of CSPs determines the behavior of caterpillars in response towards chemicals in the environment. Investigations regarding the location and function of CSPs in insect larvae are few. To date, expression of genes encoding CSPs has been studied on few species of Lepidoptera and mostly on adult specimens; few have conducted studies on the larval stage and if so, never in a tissue-specific manner (Gong et al., 2007; Jacquin-Joly et al., 2001; Liu et al., 2010; Picimbon et al., 2001). So far, the mutation of a member of the odorant-binding protein family (OBP), chemically similar to the CSP family, has been found to alter the sensitivity in the fruit fly towards a pheromone (Ebbs and Amrein, 2007; Su et al., 2009). Therefore, it is feasible that CSPs and OBPs bind nutrients, phagostimulants or may trigger immune defense mechanisms to protect the larvae against pathogens on the plant surface. Yet, it may well be, according to Dasmahapatra and collaborators (The Heliconius Genome Consortium, 2012), that this kind of proteins shuttle toxins from the plant for their accumulation in the insect body. Alternatively, they may represent additional HAMPs involved in the activation or deterrence of plant defenses against herbivory.

We have speculated that CSPs may recognize plants and/or microorganisms on the leaf surface (Celorio-Mancera et al., 2012). On the quest to challenge our hypothesis, we used a proteomics approach to test whether the levels of chemosensory proteins in salivary glands of *Vanessa cardui* (painted lady) caterpillars change depending on a variety of diet treatments. Since we aimed for the identification and relative quantification of all the proteins in the caterpillar saliva, we also paid particular attention on additional proteins involved in chemoreception, immunity and digestion, which appeared to be relevant factors due to their relative quantities in labial and mandibular glands of caterpillars (Celorio-Mancera et al., 2012). Therefore, we conducted a set of experiments to assess whether the protein levels between labial and mandibular glands changed due to: a) host plant b) a switch from artificial diet to plant material in a particular time of larval development, and c) bacteria or bacterial cells walls in the diet. In addition, since contamination of salivary gland samples with hemolymph is highly possible during dissection of these organs we considered it important to compare the proteomes of hemolymph, mandibular and labial glands from larvae reared on the same food source.

2. Methods

2.1. Insect rearing

Butterfly eggs of the species *V. cardui* were obtained from a laboratory colony and two commercial suppliers (World Wide Butterflies [www.wwb.co.uk] and Heart of England Butterflies [www.heartofenglandbutteflies.com]). Males and females were marked and placed in mating cages without including both sexes from the same origin in a given cage. Two generations were reared from this population under laboratory conditions (25 °C; LD 18:6) avoiding full-sib mating. The progeny obtained from five mating pairs from the second generation was subjected to three rearing diets following a split-brood design (Fig. 1). That is, 10 to 20 neonates from each family were transferred immediately after hatching to individual plastic cups containing either 1) soybean/wheat-based artificial diet (AD) (Stonefly "Heliothis" diet, product 38-0600, WARD's) prepared following the manufacturer's instructions, 2) leaves of marsh thistle (*Cirsium palustre*) or 3) leaves of stinging nettle (*Urtica dioica*). Leaves of either host were kept moist using a wet cotton ball at their base and replaced as needed.

2.2. Diet treatments and sample collection

Fig. 1 summarizes how the experiment was designed and conducted. In the host treatments, caterpillars fed either thistle (T) or nettle (N) from neonate stage until the time for their dissection. In treatment "AD-T", caterpillars molting into their 5th larval stage or shortly after were transferred to thistle leaves. The rest of the caterpillars reared on AD were also allocated randomly to the other three immunity treatments during or just after molting into their 5th larval stage. The control diet for the immunity treatments consisted of 3 g of artificial diet spiked with 200 μl of Luria-Bertani (LB) medium four hours before provided to the caterpillars. The treatments containing either bacterial walls (Pep) or live bacteria (Bac) were prepared exactly as the control diet but the LB medium contained peptidoglycan from Bacillus subtilis (0.08 μg/μl; Sigma−Aldrich, product no. 69554) or *Escherichia coli* bacteria (OD600 = 0.95; Invitrogen, product no. K4500). After two days of feeding, the labial and mandibular glands of 5th-instar larvae were dissected following the protocol previously described (Celorio-Mancera et al., 2012) and pooled according to gland type and biological replicate. Each feeding-treatment consisted of four biological replicates and each biological replicate per gland type (labial or mandibular) was represented by gland pairs from five individuals, one per butterfly family. The soluble protein fraction (luminal proteins) obtained per biological replicate was transferred to new tubes and further processed as described below. In order to assess