EVENT ABSTRACT

Innate immunity of insects: proteomic analysis of the relationship between Galleria mellonella and Fusarium oxysporum host-pathogen model

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The Insect immunology is an emergent field especially by the evolutive relationship with mammals, based on the counterpart molecular analogous between innate immune system, showing an adequate homologue response like the acute phase in mammals. G. mellonella larvae offer considerable advantages as an infection and virulence model because they are simple to handle and can be studied in large numbers while carrying out cost-effective experiments. Most importantly, comparing with models such as D. melanogaster or Caenorhabditis elegans species, experiments can be performed at 37°C, an optimal temperature for the vast majority of human pathogens. The remarkable advantages of this model also draw upon the innate immune response of G. mellonella larvae, which share a high degree of homology with mammalian organisms.

Fusarium oxysporum is a trans-kingdom pathogen and is well known for producing harmful secondary metabolites, called mycotoxins that cause several diseases in humans, animals, insects, and even plants. This mold is the causal agent of vascular wilt disease, which affects a wide range of plant species as tomato, but also can produce disseminated infections in human beings and immunodepressed mice. Few studies have been developed to use the larvae of G. mellonella as a heterologous host for fusaria. G. mellonella is able to resist high concentrations of F. oxysporum microconidia. When conidia are injected into the hemocoel of this Lepidopteran system, both clinical and environmental isolates of the fungus are able to kill the larvae at 37°C, although the killing occurs rapidly when incubated at 30°C

In order to validate the host-pathogen model between G. mellonella and F. oxysporum, we perform in vitro growth inhibition of the fungal pathogen, with larval hemolymph. At the other hand, we undertook proteomics research in order to understand immune response in a heterologous host when challenged with microconidia of F. oxysporum.

The strategies to reach the results were the next. 1-Larval survival test and lethal concentration of microconidia: G. mellonella larvae were arranged into 10 groups randomly selected during the last instar of the larval development and inoculated with conidia concentration (1×104, 1×105, 1×106,

1×107 and 1×108 microconidia/mL). Larvae were stored in the dark and incubated in Petri dishes at 25 and 37°C. 2- Identification and characterization of peptides by iTRAQ: the sample preparation for LC/MS/MS first used 100 µg of protein from each hemolymph sample, challenged or unchallenged with microconidia. The samples were processed using the protocol of Applied Biosystems iTRAQ 8-plex. 3-Triple TOF mass spectrometry: the multiplexed isobaric chemical tagging reaction was elaborated in the YPED proteomics laboratory, Yale Cancer Center Mass Spectrometry Resource & W.M. Peptides were separated on a Waters nanoACQUITY system via MS analysis on an AB Sciex 5600 Triple TOF mass spectrometer. Quantitation was achieved by comparing the peak areas and the resultant peak ratios for the six MS/MS reporter ions, which range from 113 to 121 Da. And 4- The statistics and bioinformatics analysis: the LC/MS/MS raw data were analyzed using the Mascot Distiller software, version 2.2, to match the peptides with a protein database using the first default parameter. The search for proteins was performed using the non-redundant NCBInr_20121109 database. Protein concentrations were determined using the ProteinPilot software, version 4.0 to infer concentrations based on the amounts of peptides assigned. To validate protein expression detected by iTRAQ, we compared these results with Real-Time-PCR (RT-PCR). Four larvae were previously treated with 106 microconidia/mL and four were untreated, both at 37°C. RNA was extracted from hemolymph and cDNA synthesis was elaborated list. RT-PCRs were developed with specific primers and the reactions were set on the Applied Biosystem device following classic quantitative PCR (qPCR) protocol.

Statistical analysis of in vitro tests showed that the challenged hemolymph inhibited the growth of the fungus, indicating that the lipids and proteins; main constituents of immunized hemolymph remain actives and prevent the normal growth of the fungus. Normality, independence and homogeneity of the data showed the rigor of experimental design. The iTRAQ approach allowed us to observe the effects of immune challenges in a lucid and robust manner, identifying 59 proteins, 17 of them involved in the immune response. Amongst 59: 20 canonical proteins from G. mellonella and 20 from the Lepidoptera species and 19 spread across other species. Some proteins were up-regulated upon immune fungal microconidia challenge when temperature changed from 25 to 37°C. After analysis of identified proteins by bioinformatics and meta-analysis, results revealed that they were involved in transport, immune response, storage, oxide-reduction and catabolism. Among of proteins of G. mellonella, actin and CREBP 3D structures were resolved using peptides matched.

We identified arylphorin, 27 kDa hemolymph protein precursor, larval hemolymph protein, hexamerin , cationic protein 8 precursor, Juvenile hormone binding protein and apolipophorins as proteins with main concentration and probably with main role. Various of these proteins were down-regulated at 25°C, but up-regulated at 37°C. Arylphorin (AAA74229) had the best peptide coverage and was likely the most abundant protein in the hemolymph, together with apolipophorins. Arylphorin is a larval storage protein (LSP) which may serve as a storage protein used primarily as a source of aromatic amino acids for protein synthesis during metamorphosis. The 27 kDa hemolymph protein precursor (P83632) has been poorly studied, and very little is known about it in Lepidoptera. Its sister protein in the hemolymph, Spz C-106, controls the

expression of the antifungal peptide Drosomycin (Drs) by acting as a ligand of Told1 and inducing an intracellular signaling pathway. Larval hemolymph protein (ABG91580), is considered another storage glycoprotein that can have different degrees of glycosylation, including different patterns and molecular sizes. The Apolipophorin (AAT76806) detected, is a lipoprotein that plays a role in lipid transport and is likely to be associated with antimicrobial activity because lipids (LPs) is secreted to prevent microbial invasions. Furthermore, the functional properties of lipophorin as a lipid carrier are well-characterized. Apolipophorin III (P80703) was identified and it was the most down-regulated protein in this study, especially after 104 microconidia/mL at 25°C. This protein seems to be unmodified under physiological conditions and all functions and effects observed must be inherent to the protein's structure. Hexamerin (AAA19801) is important members of the hemocyanin superfamily and very little is known about their characters or specific functions in G. mellonella and in general in insects, they are storage proteins with primordial functions in insect metamorphosis, but they may also have functions outside their role in storage, potentially even in immune responses. Juvenile hormone binding protein, JHBP (AAN06604) is one of the main regulators of insect development and reproduction. It is crucial for proper insect development, acting as a transporter, protector and reservoir. The expression was demonstrated to be maintained at a constant level when G. mellonella larvae were challenged with β-glucan. JHBP has been found to down-regulate transferrin and to bind to apolipophorin, arylphorin and hexamerin in the hemolymph, each of which are involved in JHBP molecular traffic.

Conclusions: the iTRAQ tool was a consistent method to detect proteins associated with the innate immune system of G. mellonella in response to infection caused by F. oxysporum. In addition, iTRAQ was a reliable quantitative proteomic approach to detect and quantify the expression levels of immune system proteins and peptides. Changes in protein expression were statistically significant, especially when temperature was increased because this was notoriously affected by F. oxysporum 104 or 106 microconidia/mL. Proteomic results were validate with rt-PCR. The results allowed us modeling the hypothesis of immune response of the insect challenged with fungus pathogen. We identify different scenarios, from the microconidia attack to the hemocoel defense.

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