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ORIGINAL ARTICLE

Binding of Orysata lectin induces an immune response in insect cells

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Abstract In mammals, plant lectins have been shown to possess immunomodulatory properties, acting in both the innate and adaptive immune system to modulate the production of mediators of the immune response, ultimately improving host defences. At present, knowledge of immunomodulatory effects of plant lectins in insects is scarce. Treatment of insect cells with the

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Orysa sativa lectin, Orysata, was previously reported to induce cell aggregation, mimicking the immune process of encapsulation. In this project we investigated the potential immunomodulatory effects of this mannose-binding lectin using *Drosophila melanogaster* S2 cells. Identification of the Orysata binding partners on the surface of S2 cells through a pull-down assay and proteomic analysis revealed 221 putative interactors, several of which were immunity-related proteins. Subsequent qPCR analysis revealed the upregulation of Toll- and immune deficiency (IMD)-regulated antimicrobial peptides (Drs, Mtk, AttA and Dpt) and signal transducers (Rel and Hid) belonging to the IMD pathway. In addition, the iron-binding protein Transferrin 3 was identified as a putative interactor for Orysata, and treatment of S2 cells with Orysata was shown to reduce the intracellular iron concentration. All together, we believe these results offer a new perspective on the effects by which plant lectins influence insect cells and contribute to the study of their immunomodulatory properties.

Key words cell culture; immune response; iron uptake; Orysata; plant lectin; pull-down

Introduction

Lectins are a complex group of carbohydrate-binding proteins that can selectively and reversibly bind to specific carbohydrate and glycoconjugate structures. These interactions mediate a multitude of biological processes, such as the regulation of the metabolism and cell growth, transport, signaling, cell-cell and cell-matrix interactions and self-recognition (Tsaneva & Van Damme, 2020). In addition, many lectins from plant and fungal origin show entomotoxic activity, raising the interest in these proteins for the development of novel pest control strategies. The effects caused by these entomotoxic lectins are as diverse as the lectins themselves. While some lectins can cause acute mortality, others may impair insect development, decrease biomass, shorten lifespan etc. (Napoleão

et al., 2019).

Since insects *N*-glycomes are dominated by high mannose-glycans (Schachter *et al.*, 2009; Walski *et al.*, 2016; Liu *et al.*, 2019; Scheys *et al.*, 2019), mannose-binding lectins received a lot of attention. One of these lectins is the *Oryza sativa* agglutinin, abbreviated as Orysata. Originally identified as a salt-inducible lectin (Zhang *et al.*, 2000), expression of Orysata was later demonstrated not to be restricted to environmental stress, suggesting participation in a broader plant defense system (de Souza Filho *et al.*, 2003). Indeed, Orysata was reported to possess entomotoxic properties against aphids (*Myzus persicae* and *Acyrthosiphon pisum*) and caterpillars (*Spodoptera exigua*) when overexpressed in tobacco plants or supplemented to an artificial diet (Al Atalah *et al.*, 2014). Although efforts have been made to gain insight into the mode of action by which lectins exert their toxicity in insects, these mechanisms were not yet completely elucidated.

In addition, plant lectins have been shown to possess immunomodulatory effects (Mishra *et al.*, 2019). They offer antimicrobial effects and effects against diseases by modulating cytokine secretion and production of other immune mediators such as reactive oxygen species to improve host defenses (Souza *et al.*, 2013; Da Silva & Correia, 2014; Coelho *et al.*, 2017). In higher animals, the immunomodulatory activity of plant lectins in various immune cells has been well documented and clearly suggests that many of these lectins have the ability to enhance phagocytic activity of immune cells and cytokine production in response to bacterial infection (Da Silva *et al.*, 2015). For example, treatment with Concanavalin A (Con A), one of the best studied plant lectins, increases the expression of various Toll-like receptors (TLRs) in murine macrophages and subsequently the secretion of pro-inflammatory cytokines and nitric oxide (Sodhi *et al.*, 2007; Kesherwani & Sodhi, 2007; Da Silva and Correia, 2014). In insects, the link between plant lectins and immune response is less obvious. One study in *Drosophila melanogaster* showed the expression of several immune-related genes, such as lysozymes, was significantly altered upon feeding with wheat germ agglutinin (WGA), which is normally seen in response to pathogens infection (Li *et al.*, 2009).

In insects, several of the immune pathways are conserved, and of these, the Toll and immune deficiency (IMD) pathways are the most extensively studied. In a typical immune pathway, one can distinguish three key steps: in the extracellular matrix, the pathogen is recognized and bound by pathogen recognition receptors (PRRs), for example, the peptidoglycan recognition proteins (PGRPs) and the Gram-negative bacteria-binding proteins 1 and 3 (GNBP1, GNBP3), whose names may cause confusion because they recognize Gram-positive bacteria and fungi (Buchon *et al.*, 2014). On the cell membrane, immune receptors such as Toll receptors receive PRRs and activate cytosol signaling. And finally, the nucleus receives stimuli to upregulate the expression of antimicrobial peptides (AMPs) (Hetru & Hoffmann, 2009). AMPs can be classified according to their target microorganisms: anti-fungus AMPs, anti-Gram-negative (G-) bacterial AMPs and anti-Gram-positive (G+) bacterial AMPs. In addition, they can also be classified according to specific immune pathways by which they are activated, such as IMD- or Toll-activated AMPs (Imler & Bulet, 2005). Furthermore, several immune pathways, such as the c-Jun N-terminal kinases (JNK) pathway, can regulate the production of pro-apoptotic factors which may promote programmed cell death (Georgel *et al.*, 2001). For example, the Drosophila AMP defensin can promote tumor cell apoptosis triggered by Egr, a tumor necrosis factor produced by tumor cells through the JNK pathway (Freeman *et al.*, 2019).

Physiological changes, e.g. in iron homeostasis, are also crucial in the immune response (Nairz *et al.*, 2014). In higher animals, cytokines can affect iron uptake leading to ion retention in macrophages, limiting the availability of iron for the extracellular pathogens and inducing inflammation. On the other hand, iron affects the innate immune response, causing iron-loaded macrophages to lose their ability to kill intracellular pathogens (Nairz *et al.*, 2014). In addition, iron deficiency can also cause apoptosis (Koc *et al.*, 2006), which is interesting because some reports suggested that lectins can decrease iron uptake (Hisayasu *et al.*, 1992; Dabravolski & Kavalionak, 2019). For example, the iron storage protein ferritin was identified as one of the interaction partners of the *Galanthus nivalis* agglutinin (GNA) in *Nilaparvata lugens* (Du *et al.*, 2000).

Previously, treatment of insect cells with Orysata was shown to cause cell aggregation, an effect mimicking the immune process of encapsulation, suggesting possible immunomodulatory properties of this lectin (Chen *et al.*, unpublished). In this project we investigated the immunomodulatory effects of the Orysata lectin in more detail using *D. melanogaster* S2 cells, a cell line of embryonic origin. Insect cell lines are a valuable tool for the study of cellular mechanisms of lectin interactions (Smagghe *et al.*, 2009). After confirming that Orysata treatment did not cause acute cell toxicity in S2 cells, the interaction targets for Orysata on the surface of these cells were identified. A pull-down analysis with a membrane cell extract of S2 cells as prey led to the identification of a potential link with the immune system and iron transport. Indeed, detailed investigation revealed that Orysata may trigger insect immunity and influence iron uptake. This study offers a new perspective on the interactions between plant lectins and insect cells, specifically on the immunomodulatory effects of such lectins.

Material and methods

Insect cell maintenance

S2 cells from *D. melanogaster* were maintained in SF900 medium (Thermo Fisher, Waltham, MA, USA) supplemented with 10% Gibco™ fetal bovine serum (FBS) (Thermo Fisher), and sub-cultured as described (Wang *et al.*, 2020).

Production, purification and FITC labeling of recombinant Orysata

A detailed protocol for the production and purification of recombinant Orysata purification was described by Al Atalah *et al.* (2011). In brief, the coding sequence of *Orysata* was ligated into the pPICZBα vector containing a secretion signal, a c-myc epitope and a poly-histidine tag. The recombinant vector, pPICZBα::Orysata, was transformed into the yeast *Pichia pastoris* strain X33.

The recombinant strain, X33::pPICZBα::Orysata, was induced with methanol to express Orysata for 48 h, after which the culture medium was harvested and the proteins isolated by ammonium sulfate precipitation. After dialysis, the precipitated proteins were purified by ion exchange and Ni^{2+} affinity chromatography. After confirming Orysata by western blot, purified proteins were dialyzed in PBS and filter sterilized.

The recombinantly produced Orysata was labeled with fluorescent FITC using the FITC labeling kit (Sigma Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. In brief, 2 mg of recombinant Orysata was dissolved in 0.5 mL of 0.1 mol/L sodium carbonate pH = 10. In total 50 μL of fresh 4 mg/mL FITC (in pure DMSO) was added to the Orysata solution in 10 steps of 5 μL each and, in between, the mixture was kept at 4 °C and mixed gently. The mixture was incubated for 8 h at 4 °C in the dark. Then about 5 μL of 5 mol/L NH_4Cl was added to a final concentration of 50 mmol/L, and the mixture was incubated for 2 h at 4°C. The free dye was removed by overnight dialysis in PBS pH = 7.4 at 4°C. The concentration and FITC/protein (F/P) ratio were measured using the following equations, the F/P value should be around 0.3~1.0.

$$Molar\ F/P = \frac{MW}{389} \times \frac{(A_{495}/195)}{([A_{280}-(0.35\times A_{495})]/E^{0.1\%})} = \frac{A_{495}\times C}{A_{280}-(0.35\times A_{495})}$$ with

$$C = \frac{MW\times E^{0.1\%}_{280}}{389\times 195}$$

The FITC-labeled Orysata concentration was calculated with following equation:

$$[FITC\text{-}Orysata\ ](mg/ml) = \frac{[A_{280}.(0.35\times A_{495})]}{0.944}$$ with 0.944 the A_{280} of recombinant Orysata at a concentration of 1 mg/mL and 0.35 the contribution factor of FITC in the A_{280}.

Cell growth analysis

When healthy cells reached 90% confluency, they were collected and diluted to a density of 1 × 10^5 cells/mL with fresh culture medium. In CELLSTAR® Cell-Repellent Surface 24-well plates (Greiner

Bio-One, Vilvoorde, Belgium), 400 μL of the Orysata-mixed culture medium was added to each well and mixed with 200 μL of diluted cells (20 000 cells per well). After dilution, the Orysata concentration was 3 μmol/L. Relative cell numbers were measured using the PrestoBlue™ reagent (Thermo Fisher) after incubation for 0, 24, 48 and 72 h according to the manufacturer's instructions.

Extraction of membrane proteins, pull-down assay and proteomic analysis

When the cells reached 90% confluence, they were washed twice with PBS. Approximately 10^7 cells were solubilized in suspension buffer [100 mmol/L Tris-HCl (pH 7.2) + 150 mmol/L NaCl + protease inhibitor (Thermo Fisher)] and lysed by three freeze-thaw cycles (−80 °C and room temperature). The membrane and cytosolic fraction were separated by centrifugation at 13 000× *g* for 25 min at 4 °C. The supernatant, containing the cytosolic fraction, was discarded. The pellet, containing the membrane fraction, was washed twice with 500 μL of cold suspension buffer and centrifuged at 13 000× *g* for 5 min at 4 °C. Finally, membrane proteins were extracted by dissolving the pellet in 10–50 μL RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Fisher) and diluted in 500 μL of wash buffer (10 mmol/L Tris-HCl pH 7.2, 150 mmol/L NaCl, 0.5 mol/L EDTA). The protein concentration was quantified using the RCDC protein assay (Bio-Rad, Hercules, CA, USA). For the pull-down assay, equilibrated myc-trap beads were first incubated with 100 μg of Orysata (in wash buffer) for 1 h on a rotator at 4 °C. After a short centrifugation, the Orysata-beads were washed twice to remove unbound lectin and 1 mg of S2 membrane proteins was added to the beads and incubated for 1 h at 4 °C. The protein-Orysata beads were collected and washed twice before resuspension in 40 μL of trypsin digestion buffer (20 mmol/L Tris-HCl pH 8, 2 mmol/L $CaCl_2$). 10 μL of the beads was used for SDS-PAGE and silver staining to confirm the binding of proteins. The remainder of the sample was processed for LC-MS/MS analysis as described by Scheys *et al.* (2018). In brief, trypsin digested samples were dried and re-dissolved in 20 μL loading solvent A [2% acetonitrile (ACN); 0.1% TFA in ddH_2O], 5 μL of which was

injected in an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive mass spectrometer (Thermo Fisher) for LC-MS/MS analysis. Spectra were searched with the MaxQuant software (v1.6.3.4) (Tyanova *et al.*, 2016a) against the Uniprot reference proteome of *Drosophila melanogaster* (taxid 7227, database release of July 2019 containing 21 975 protein sequences, www.uniprot.org) supplemented with the recombinant Orysata lectin protein sequence. The data was further processed in Perseus (v1.6.14.0) (Tyanova *et al.*, 2016b). In short, the protein intensities were log2 transformed and proteins only identified by site were removed as well as the reversed hits and potential contaminants. The triplicate experiments were grouped and proteins with less than three valid values in at least one group were removed. After imputation of the missing values, the protein intensities of the Orysata and Control samples were compared using a two-sample *t*-test (FDR = 0.01 and S_0 = 1). A total of 221 proteins, which were significantly enriched in the Orysata samples, were selected for further bioinformatics analysis. Several tools were used to analyze these proteins, including signal peptide prediction (SignalP 5.0, Almagro Armenteros *et al.*, 2019), transmembrane domain prediction (TMHMM, Krogh *et al.*, 2001) and glycosylation prediction (NetNGlyc, Gupta *et al.*, 2004). Gene ontology (GO) analysis was done with PANTHER (http://www.pantherdb.org/).

Transcript profiling of response in S2 cells after exposure to Orysata

This assay followed the same methods as described for the cell growth test, 200 μL of cells (1×10^5 cells/mL) was added to 400 μL of Orysata-mixed culture medium. After incubation for 48 h, cells were harvested and the total RNA was extracted using the RNeasy® Mini Kit (QIAGEN, Hilden, Germany). Afterwards, cDNA was synthesized from 1 μg of purified RNA by SuperScript® IV Reverse Transcriptase (Invitrogen, Merelbeke, Belgium) in a 20 μL-reaction system and then diluted 20-fold as template for quantitative PCR (qPCR). Every 20 μL-qPCR reaction system included 8.0 μL of cDNA template, 1 μL of forward primer, 1 μL of reverse primer and 10 μL of GoTaq® qPCR Master Mix

(Promega, Madison, WI). Reaction cycles were: 95 °C for 3 min, then 39 cycles of 95 °C for 10 s and 55 °C for 30 s. Relative expression was analyzed by qBase software. For internal reference, SdhA and RPL32 were selected. The primers used are listed in Table S1.

Measurement of iron inside S2 cells after exposure to Orysata

Iron concentration was quantified using the colorimetric ferrozine-based assay as described by Riemer *et al.* (2004). In a 24-well plate, 20 000 logarithmic growing S2 cells were seeded and grown for 24 h to allow attachment to the plate. These cells were subsequently mixed with Orysata to a final concentration of 3 μmol/L and incubated for 1 h at 27 °C. PBS was used as a control. Subsequently, 60 μL of 1 mmol/L sterilized iron solution (ferric ammonium citrate, FAC (Sigma Aldrich)) was added and cells were incubated for 48 h. After measuring the cell numbers in each well using the PrestoBlue assay, the culture medium was removed, and the cells were washed twice with 600 μL of PBS. For cell lysis, 250 μL of RIPA solution was added to the cells and incubated for 10 min on ice. The total protein concentration of each sample was measured using the RCDC protein assay (Bio-Rad). To determine the iron concentration 100 μL of cell lysate was mixed with 100 μL of RIPA buffer and 100 μL of iron release reagent [1 : 1 (v : v) = 1.4 mol/L HCl : 4.5% $KMnO_4$]. The mixture was heated at 60 °C for 2 h in a fume hood (to remove the Cl_2 produced) and then cooled down to room temperature. After centrifugation at 16 000 *g* for 1 min at room temperature, 250 μL of the supernatant was transferred to each well of a transparent flat 96-well plate. 30 μL of iron detection solution (6.5 mmol/L ferrozine, 6.5 mmol/L neocuproine, 2.5 mol/L ammonium acetate, 1 mol/L ascorbic acid) was quickly added to each sample well and incubated for 30 min in the dark. Afterwards, the absorbance at 550 nm was measured to calculate the iron concentration using a standard curve and calibrated to the cell number (nmol/L iron per 10 000 cells).

Results

Orysata inhibits proliferation of insect cells

During the timespan of the experiment, treatment of S2 cells from *D. melanogaster* with 3 μmol/L Orysata did not lead to acute cell toxicity, there were no visual signs of cell lysis (cell debris) apparent. However, the formation of cell aggregates was observed after treatment of S2 cells with Orysata (Fig. 1A). In addition, while under control conditions the cell number steadily increased, proliferation in Orysata treated cells was inhibited, suggesting a biostatic effect of the lectin (Fig. 1B).

Orysata binds to the cell surface and is slowly internalized

To study the location of the interaction between Orysata and insect cells, the lectin was fluorescently labeled to follow its binding to the cell surface and internalization into the cells. Confocal microscopy analysis of S2 cells exposed to FITC-labeled Orysata showed abundant binding of the lectin to the cell surface within 20 minutes of incubation (Figs. 2A and 2B). After 4 h, Orysata was found to be internalized into the S2 cells, (Figs. 2C–F).

Identification of interaction partners for Orysata on the S2 cell surface

To gain insight into the molecular processes influenced by Orysata binding, its interaction partners on the surface of S2 cells were identified. Therefore, a pull-down assay was performed using recombinant Orysata as bait and an extract of S2 membrane proteins as prey. Proteomic analysis identified a total of 1 881 proteins from both the control and pulldown samples (Table S2; Fig. S1A–B), of which 221 proteins were significantly enriched in the Orysata pull-down sample (Table S3). These proteins were analyzed for the presence of a signal peptide or a transmembrane domain to confirm their surface localization. About 67% of these proteins contain one or more transmembrane (TM) domains, and 68% has a signal peptide (Table S3; Fig. S1C–D). With a total of 95% of the proteins carrying either a TM domain, signal peptide or both, the results suggest an enrichment in

membrane-bound and/or secreted proteins.

The functionality of these putative interactors was explored using GO analysis (Fig. 3; Table S4). Out of 221 identified proteins, 148 were mapped with GO (http://www.pantherdb.org/) items (Table S4). The Cellular Component GO analysis confirmed the enrichment of membrane-bound and secreted proteins, with membrane (GO:0016020), cell periphery (GO:0071944) and extracellular region (GO:0005576) being the GO terms linked to tens of proteins (Fig. 3A, Table S4). For example, integrin alpha-PS3 (O44386) and contactin (Q9VN14) are both located on the plasma membrane (GO:0005886, subgroup of membrane (GO:0016020)), however, the membrane localization of integrin alpha-PS3 depends on its TM domain, while for contactin it depends on its GPI anchor. Based on GO terms, the putative interactors can play a role in diverse metabolic processes, such as the protein metabolic process (GO:0019538), carbohydrate derivative metabolic process (GO:1901135) and phospholipid metabolic process (GO:0006644) (Fig. 3B, Table S4). In these processes their molecular function includes catalytic activity (GO:0003824, GO:0140096, GO:0016740) and binding (GO:0097159, GO:1901363, GO:0003676) (Fig. 3C; Table S4). GO terms overrepresented in the putative interactors of Orysata compared to the *D. melanogaster* reference are represented in Figure 3.

Interesting, the GO analysis of the reactome pathways revealed an enrichment of terms related to the immune system (R-DME-168256), with more than 20 proteins belonging to the innate immune response (GO:0045087) (Fig. 3D; Table S4). These Orysata binding proteins include, amongst others, Toll/IMD components required for the activation of AMP production. For example, the gram-negative bacteria-binding protein 2 (GNBP2, Q9VVR4) which can recognize pathogens and activate the serine proteases (M9PCH6, X2J9Z1, Q9VDT5, Q9VW19, Q6AWJ5). These serine proteases mediate a signaling cascade, finally leading to the processing of Spaetzle (P48607), the ligand for the Toll receptor (Toll isoform C, A0A0B4KHY4). In addition, the iron binding protein transferrin 3 (Tsf3), with a key role in the iron homeostasis and annotated with GO terms linked to the immune

reactome pathways, was identified as a putative interactor of Orysata (Table S3).

Orysata treatment leads to differential expression of immune-related genes

The identification of immune-related proteins as putative interactors for Orysata suggested immunomodulatory effects of this lectin. Therefore, transcript levels for several receptors, signal transducers and effector genes of the Toll and IMD pathways were analyzed in S2 cells treated with Orysata. We investigated the expression of Toll-related regulators such as the Gram-negative bacteria-binding proteins 1 and 3 (GNBP1, GNBP3), the peptidoglycan recognition protein SA (PGRP-SA), the serine proteinase ModSP and the spatzle process enzyme (SPE), as well as the cell surface Toll receptors 5 and 9 (Toll-5 and Toll-9). In addition, the transcript levels of IMD related regulators, including the PGRP-LC and relish (Rel), as well as of effector AMPs belonging to the Toll and/or IMD pathways (drosomycin (Drs), IMD-regulated attacin A (AttA) and diptericin (Dpt), and Toll/IMD-coregulated defensin (Def) and metchnikowin (Mtk)) were also investigated using RT-qPCR. Furthermore, a JNK bypass mediator, the head involution defective (Hid) was also analyzed. In general, an upregulation (0.1–14 fold increase) of the transcript levels for the selected immune related genes was observed 48 h after Orysata treatment, except for SPE and Def (Fig. 4).

Orysata treatment reduces iron uptake

The iron-binding glycoprotein, transferrin 3 (Tsf3), was identified as a potential interactor for Orysata from the pull-down assay (Table S3). To investigate a potential link between Orysata treatment and iron homeostasis in insect cells, iron uptake was measured in S2 cells treated with 3 μmol/L Orysata. After 48 h, the cellular iron concentration per 10 000 cells was significantly reduced by 60% in Orysata treated S2 cells (Fig. 5). Subsequent analysis of the transcript levels for Tsf3 in response to Orysata treatment revealed an upregulation after 48 h of treatment (Fig. 5). In contrast,

the expression of another iron transporter, Tsf1, was not significantly altered by the Orysata treatment. The transcript levels of the iron storage proteins ferritin 1 and ferritin 3 were also observed to be upregulated, but ferritin 2 was not.

Discussion

In mammals, plant lectins are known as potent immunomodulatory agents, able to act in both the innate and adaptive immune system, where they modulate the production of cytokines and other mediators of the immune response, ultimately improving host defenses (Souza *et al.*, 2013; da Silva & Correia, 2014; Coelho *et al.*, 2017; Jandu *et al.*, 2017; Mishra *et al.*, 2019). Despite being the largest taxa on earth, the immunomodulatory effects of plant lectins are understudied in insects. As many plant lectins have entomotoxic properties, lectin research is mainly focused on the exploitation of these properties in novel pest control strategies and knowledge on their immunomodulatory effects in insects is scarce.

Orysata, a mannose-specific lectin from *O. sativa* (rice) (Al Atalah *et al.*, 2014), did not cause acute cellular toxicity to *D. melanogaster* S2 cells. However, treatment with this lectin induced the formation of cell aggregates, which was accompanied with an inhibition of cell proliferation. These effects resemble to the immune effect of encapsulation, a process in which hemocytes gather together and enclose a pathogen by making a capsule-like structure, thereby limiting its mobility, development and/or proliferation (Ishihara *et al.*, 2017), suggesting this lectin might have immunomodulatory effects on insect cells. The immunomodulatory activity of plant lectins is often associated with their interaction with glycan moieties present on cell surfaces (reviewed by Chen *et al.*, 2021). Such interactions can result in a signal transduction which triggers the effector mechanisms involved in the immune response (reviewed by Souza *et al.*, 2013; Chen *et al.*, 2021). In this study Orysata was observed to readily bind to the cell surface of *D. melanogaster* S2 cells, possibly through interaction with the glycans of the glycoproteins on the cell surface.

To identify the receptors on the surface of S2 cells and elucidate the molecular mechanisms by which Orysata exerts its effects, a pull-down assay was performed to identify potential Orysata interactors. In addition to the integral membrane proteins, this analysis identified several secreted proteins, as suggested by the presence of a signal peptide. However, it is known that secreted proteins can associate with the cell membrane, amongst others through interactions with cell surface proteins or membrane anchors. For example, spaetzle (P48607), captured by Orysata, is a secreted protein that interacts with the Toll receptor on the cell surface. Similarly, contactin, a well-studied secreted protein, locates to the cell surface because of its GPI anchor (reviewed by Karagogeos, 2003). Among the 221 Orysata enriched proteins there are many proteins that directly participate in the cellular immune response, including extracellular proteins like the Toll receptor and the Gram-negative binding protein (GNBP2). This indicates a potential immunomodulatory activity of Orysata, similar to the previously reported WGA-induced immune responses in the Drosophila larval midgut (Li *et al.*, 2009). Therefore, the transcriptional expression of Toll/IMD-immune pathway-related genes, including PRRs, serine proteinases, membrane receptors and antimicrobial peptides (AMPs), was analyzed after treatment of S2 cells with Orysata. The tested Toll- and IMD-regulated PRRs, such as GNBPs and PGRPs, as well as AMPs (Drs, Mtk, AttA and Dpt) (Fig. 4) all showed an upregulation of their transcript levels 48 h after Orysata treatment, except for the Def. Activation of IMD is also proven by upregulation of its marker mediator Relish (Rel) and the IMD-JNK pathway effector Hid.

Iron is an essential component of many proteins and enzymes. Cellular and systemic iron homeostasis is strictly controlled through finely tuned complex mechanisms, which can be modulated as part of the immune response, for example to reduce the availability of iron to extracellular pathogens (Nairz *et al.*, 2014). Transferrin, as well as the ferritins, are proteins important for iron homeostasis, which are also crucial in the sequestration of iron as part of the immune reaction. Tfs3 was here identified as a putative target of Orysata, suggesting that Orysata

might have an effect on the immune related cellular iron homeostasis. While it was shown that treatment with Orysata reduced the iron levels, the underlying mechanism is not yet elucidated. In a previous study, it was shown that *Sambucus sieboldiana* agglutinin (SSA) binding to transferrin can block its recognition to a specific antibody (Ito *et al.*, 2018). This suggests a possibility that binding of Orysata to Tsf3 might block iron transportation, leading to reduced intracellular iron levels. However, the iron loss can also be the result of the Orysata induced immune response. When analyzing transcript levels for Tsf1, Tsf3 and ferritins in S2 cells treated with Orysata, an upregulation of transcription of Tsf3, ferritin2 and ferritin3 was observed, this could be a compensatory mechanism in response to the decreased intracellular iron levels or can be part of the immune response. The latter agrees with mutant experiments indicating that transferrin is co-regulated by the Toll and IMD pathway (De Gregorio *et al.*, 2002). In addition, infection studies also proved that the upregulation of transferrin participates in the immune response. For example, after treatment with heat-killed bacteria, mosquito cell cultures respond by upregulating transferrin (Yoshiga *et al.*, 1997; Yoshiga *et al.*, 2001). Iron sequestration by these iron binding proteins is an important immune response which limits the iron bioavailability for pathogen growth (Parrow *et al.*, 2013). These results suggest that Orysata has immunomodulatory properties in insect cells regulating the immune pathways and iron homeostasis.

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Supporting Information

Fig. S1 Analysis of S2 membrane proteins binding to Orysata. A, silver staining of Orysata pulled-down S2 membrane proteins. Red arrows indicate the Orysata lectin, with one band glycosylated (Al Atalah *et al.*, 2011). B, Visualization of the *t*-test results as a Volcano plot. The purple squares outside the right limit (FDR: 0.1 and S0: 1) represent protein groups that are significantly enriched in the Orysata pull-down. C-D, From the 221 significantly Orysata-enriched proteins, 66% contained at least 1 predicted transmembrane (TM) domain (C) and 68% contained a predicted signal peptide (D). Analysis was, respectively, performed in THMHH and SignalP.

Table S1 Primers used in this study.

Table S2 Identified proteins in the pull down assay.

Table S3 Putative interactors for Orysata.

Table S4 GO analysis.

Figure legends

Fig. 1 Viability of S2 cells. A, The lack of visible signs of cell debris suggests treatment with 3 μmol/L Orysata did not cause cell toxicity. However, treatment of Orysata induced the formation of cell aggregates. S2 cells were observed under a light microscope with a 10× lens. B, S2 cell growth after exposure to 3 μmol/L Orysata. Numbers above the bars indicate the *P*-value of a *t*-test compared to

the PBS control for each time point, n = 4. Asterisks indicate the significance levels according to the p-values, ns indicates no significance.

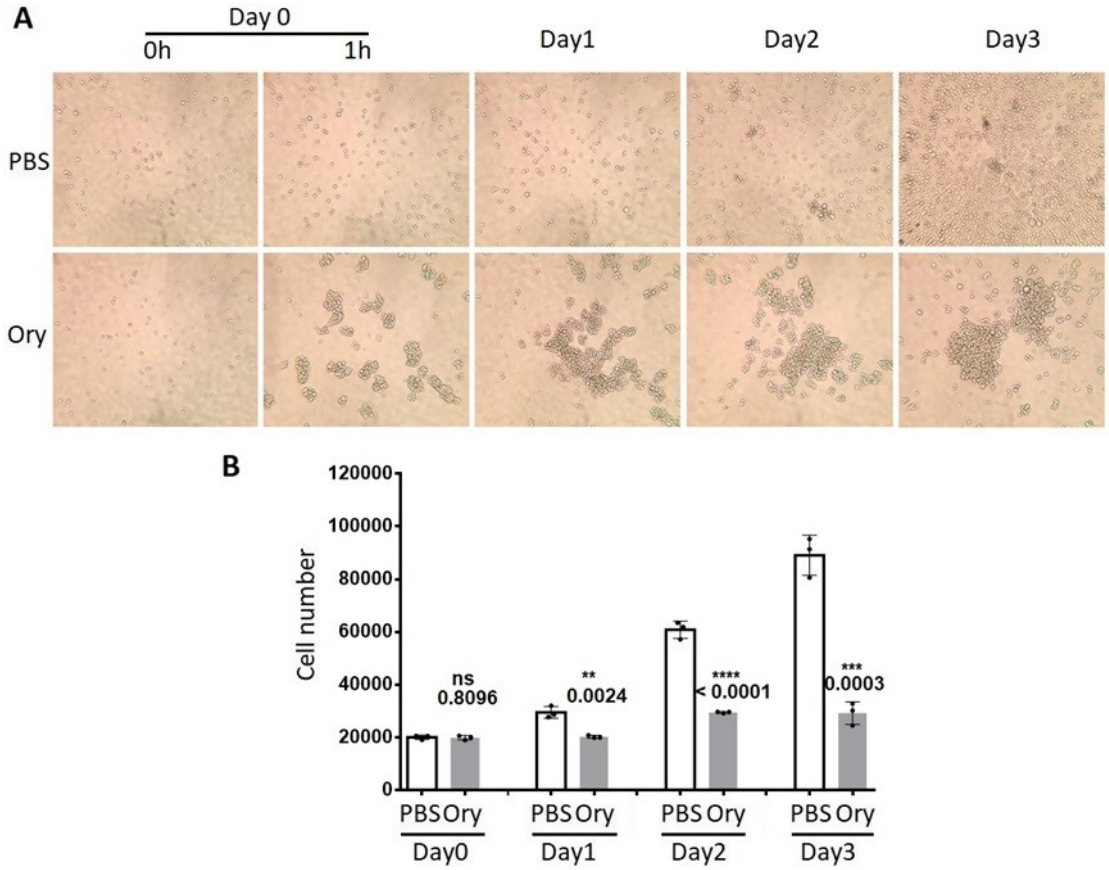

Fig. 2 Localization of Orysata in S2 cells. Transmission image (A) and FITC-fluorescence (B) of S2 cells at 20 min after treatment with 3 μmol/L of FITC-labeled Orysata. C–F, at 4 h of incubation with 3 μmol/L of FITC-Orysata. C, nucleus detected by Hoechst stain. D, FITC-Orysata. E, cell membrane stained by CellMask^{TM} Deep Red. F, merged signal of Hoechst, CellMask and FITC. All scale bars indicate 10 μm.

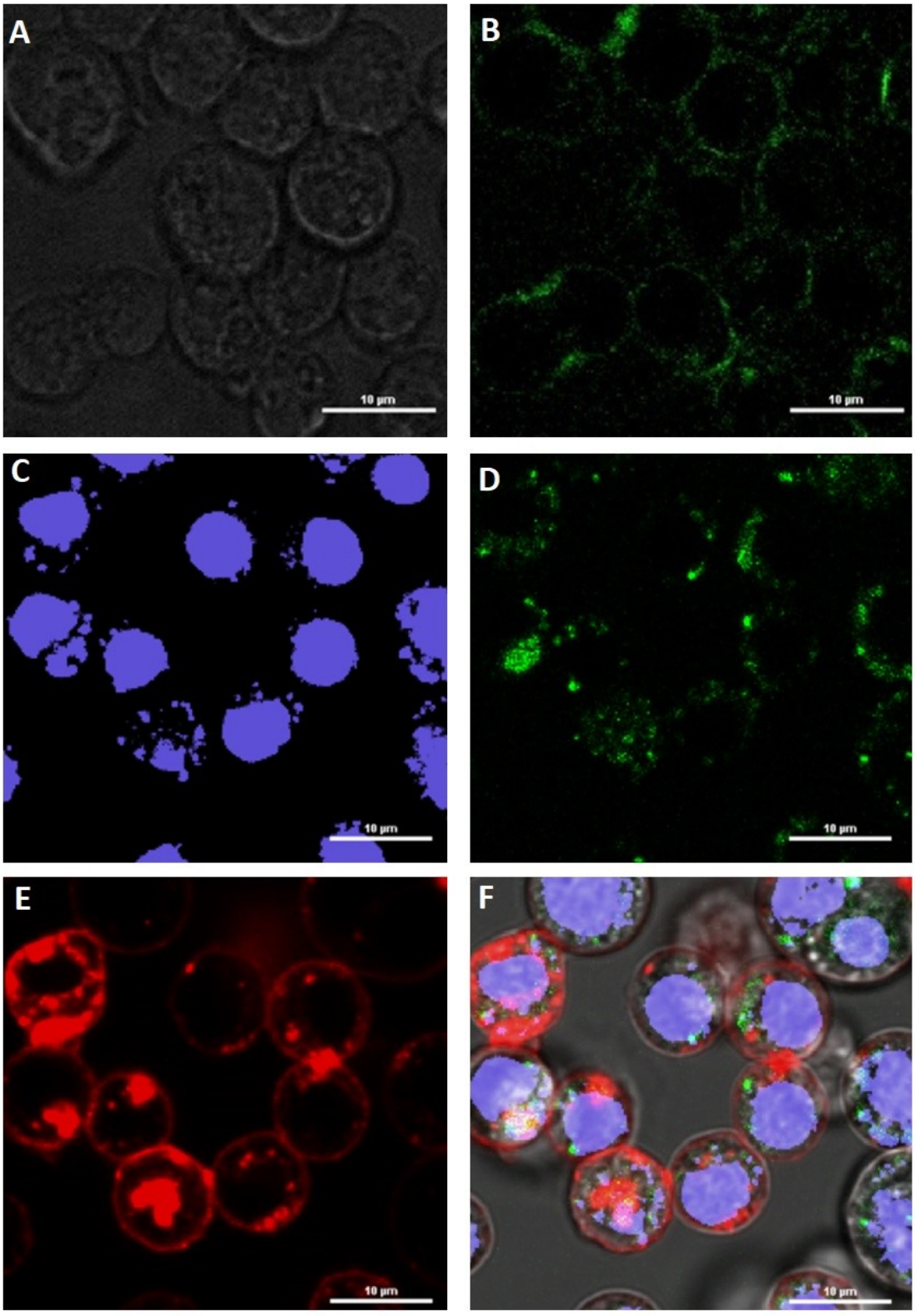

Fig. 3 Gene ontology of the 221 Orysata-binding proteins found in membrane extracts of S2 cells after a pull-down assay. 148 out of 221 proteins are mapped on GO database and used for overrepresentation test. All listed items are significantly overrepresented by FISHER test ($P > 0.05$)

compared to the *Drosophila melanogaster* reference (Table S4). A, Cellular component GO items. B, Biological process GO terms. C, Molecular function GO terms. D, Reactome pathways terms.

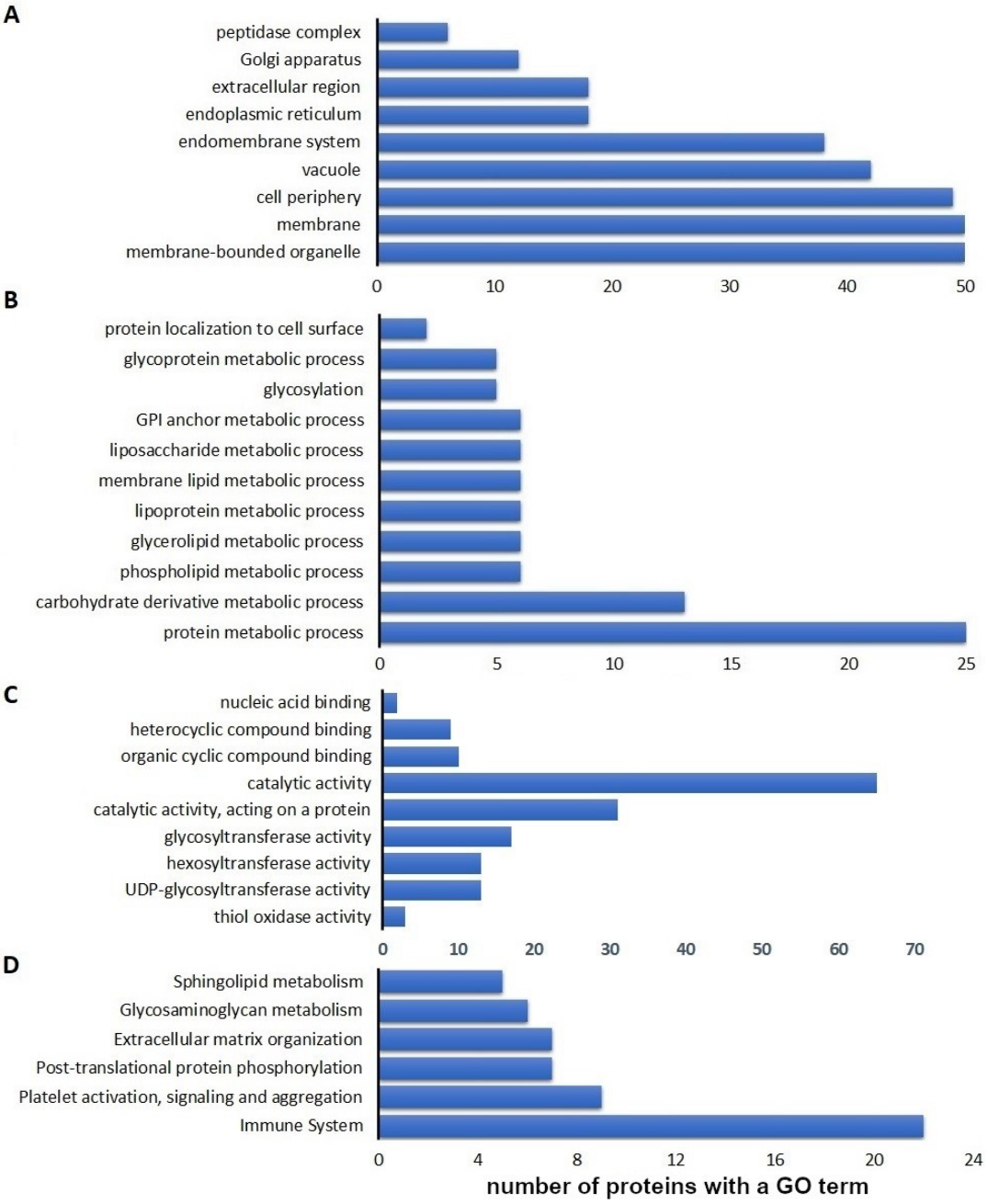

Fig. 4 Orysata treatment influences the expression of immune-related genes. The illustration shows the classical immune pathway of insects (redrafted from Buchon *et al.*, 2014). Transcription levels of immune-related genes are measured by RT-qPCR in S2 cells after 48 h exposure to 3 μmol/L Orysata. Numbers above the bars indicate the *p*-value of a *t*-test compared to the PBS treatment, $n = 4$.

Asterisks indicate the significance levels according to the *P*-values, ns indicates no significance.

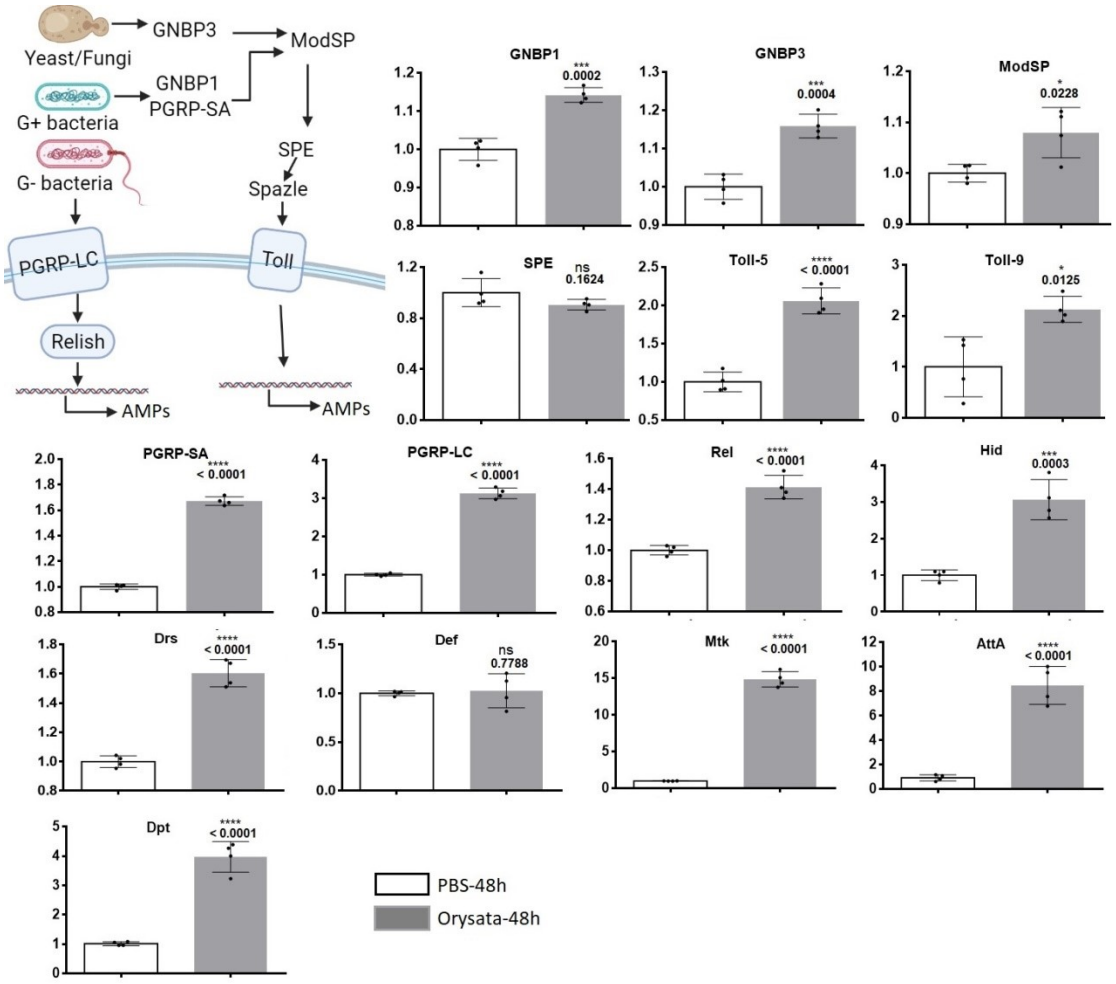

Fig. 5 Orysata treatment reduces cellular iron content. Iron concentration per 10 000 S2 cells and mRNA transcript levels for iron related genes were measured after exposure to 3 μmol/L Orysata for 48 h or mock treatment (PBS). Numbers above the bars indicate the *P*-value of *t*-test compared to the PBS treatment of each time point, n = 4.