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Internalization of a sunflower mannose-binding lectin into phytopathogenic fungal cells induces cytotoxicity

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ABSTRACT

Lectins are carbohydrate-affinity proteins with the ability to recognize and reversibly bind specific glycoconjugates. We have previously isolated a bioactive sunflower mannose-binding lectin belonging to the jacalin-related family called Helja. Despite of the significant number of plant lectins described in the literature, only a small group exhibits antifungal activity and the mechanism by which they kill fungi is still not understood. The aim of this work was to explore Helja activity on plant pathogenic fungi, and provide insights into its mechanism of action. Through cellular and biochemical experimental approaches, here we show that Helja exerts an antifungal effect on *Sclerotinia sclerotiorum,* a sunflower pathogen. The lectin interacts with the fungal spore surface, permeabilizes its plasma membrane, can be internalized into the cell and induces oxidative stress, finally leading to the cell death. On the other hand, Helja is inactive towards *Fusarium solani,* a non-pathogen of sunflower, showing the selective action of the lectin. The mechanistic basis for the antifungal activity of an extracellular jacalin lectin is presented, suggesting its initial interaction with fungal cell wall carbohydrates and further internalization. The implication of our findings for plant defense is discussed.

1. Introduction

Plants have naturally developed different mechanisms to counteract the attack of phytopathogenic fungi, including antifungal proteins and peptides. These have been isolated from diverse plant species, and can be classified according to their structure and/or function into different groups including chitinases, glucanases, thaumatin-like proteins, defensins, lipid transfer proteins, lectins, peroxidases, protease inhibitors, among others (Wong et al., 2010; Yan et al., 2015). The lectins have the unique ability to recognize and reversibly bind specific carbohydrate ligands without any chemical modification; this feature distinguishes lectins from other carbohydrate binding proteins and enzymes (Peumans and Van Damme, 1995). Plants were the first discovered source of lectins, and although these proteins are ubiquitously distributed in nature, plants remain their most frequent source due to both ease of extraction and the relatively high yields that can be obtained (Lam and Ng, 2010; Dang and Van Damme, 2015). Although currently still under study, it is generally accepted that plant lectins can play a defense role recognizing the pathogens and initiating the stress response through protein-carbohydrate interactions (De Schutter and Van Damme, 2015). Related to their binding-ability to glycoconjugates, lectins exhibit a diversity of activities including antibacterial, anti-insect, antitumor, immunomodulatory, HIV-1 reverse transcriptase inhibitory activities, which make them valuable tools in different practical applications (Lam and Ng, 2010; Dang and Van Damme, 2015; Lagarda-Diaz et al., 2017; Poiroux et al., 2017). Despite the large numbers of lectins that have been purified and characterized, only a few of them displayed antifungal activity (Silva et al., 2014; Wu et al., 2016; Neto et al., 2017). Carbohydrates present on the fungal cell wall represent the main structures engaged in primary interaction with host cells and constitute ideal targets for antifungal protective intervention. Thus, the lectins are particularly attractive since they can affect the plant-pathogen interaction at the first point of contact. It is generally accepted that plant lectins are unable to bind glycoconjugates present on fungal membranes or penetrate into the fungal cytoplasm owing to the cell wall barrier (Wong et al., 2010; Dang and Van Damme, 2015). Hence, it is unlikely that lectins directly interfere with fungal growth by altering the structure and/or permeability of their membranes. However, there may be indirect effects produced by the binding to the carbohydrates on the surface of the fungal cell wall (Wong et al., 2010).

We have previously isolated a sunflower mannose-binding lectin belonging to the jacalin-related lectins (JRL) family, which was called Helja (*Helianthus annuus* jacalin) (Pinedo et al., 2012). Helja was initially detected through a proteomic study of sunflower seedlings apoplast and its putative identification as mannose binding jacalin was taken as an advantage to purify it by D-mannose affinity

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chromatography. Biochemical and cellular approaches demonstrated the extracellular localization of Helja in sunflower seeds and seedlings (Pinedo et al., 2012). The characterization of the biological activity revealed that the protein displayed agglutination capacity on *Saccharomyces cereviceae* cells and antifungal activity on human pathogens of *Candida* genus (Regente et al., 2014). Although the physiological role of jacalin-related lectins is still not understood, in general, it has been shown to be involved in resistance to abiotic and biotic stress (Xiang et al., 2011; Song et al., 2014; Esch and Schaffrath, 2017). For instance, the rice protein OsJRL is up-regulated in response to salt, drought, cold, and heat stress (He et al., 2017). The jacalin-related lectin RTM1 restricts the long distance movement of tobacco etch virus in *Arabidopsis thaliana* (Chisholm et al., 2000). The apoplastic localization of Helja and its inhibitory activity on a human pathogenic fungus model suggest that the lectin could be a component of the plant defense system towards the fungal attack. The aim of this paper was to explore the activity of Helja on plant pathogenic fungi, and provide mechanistic insights on its mode of action. The research design was based on the evaluation of Helja biological activity on *Sclerotinia sclerotiorum*, the causal agent of sunflower head rot. In addition, the antifungal activity of Helja on *Fusarium solani,* a non-pathogen of sunflower, was also evaluated. The comparison of the results obtained after Helja treatments on the two fungus species, both from the point of view of its mechanism of action as well as from its role in the interaction between microbial and host plant cell will be discussed.

2. Materials and methods

2.1. Biological material

Sunflower seeds (*Helianthus annuus* L., line 10347) were provided by Advanta Semillas SAIC, Venado Tuerto, Argentina. *Sclerotinia sclerotiorum* (Lib.) de Bary ascospores from a local virulent isolate were a gift of M. E. Bazzalo (Advanta Semillas SAIC, Centro Biotecnológico Balcarce, Argentina) and were collected from Petri dishes containing imprints of apothecia. *Fusarium solani* f. sp. *eumartii*, isolate 3122, (INTA Collection, Balcarce, Argentina) was kindly provided by Dr. C. Casalongué. *F. solani* was grown at 25 °C on potato dextrose agar (PDA) plates and spores were collected from cultures by suspension in sterile water.

2.2. Helja purification

Sunflower seeds were imbibed for 16 h and subjected to the extraction of the extracellular fluids (EF) by a standard infiltration-centrifugation procedure (Regente et al., 2008). Briefly, seeds were immersed in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% 2-mercaptoethanol and subjected to three vacuum pulses of 10 s, separated by 30 s intervals. The infiltrated seeds were recovered, dried on filter paper, placed in filters and centrifuged for 20 min at 400*g* at 4 °C. The EF was recovered in the filtrate and subjected to Helja purification according to Pinedo et al. (2012), with some modifications. The EF was loaded on a 1 ml D-mannose-agarose resin (Sigma M6400) equilibrated with 50 mM HCl-Tris pH 7.5, 100 mM NaCl (buffer A). Non-bounded proteins were washed with buffer A before the elution of retained proteins with 0.2 M mannose in the same buffer. The eluted fraction was exhaustively dialyzed against distilled water to allow the release of mannose from the protein fraction.

2.3. Microscopic antifungal activity assays

Fresh spores from *S. sclerotiorum* and *F. solani* were collected in sterile water and subjected to counting in a Neubauer chamber for further calculation of appropriate dilutions. The antifungal activity was evaluated on micro slides in a final volume of 20 µl containing the protein sample (0.01; 0.05; 0.1 and 0.2 µg µl^{-1}), 1 × 10^5 spores and 4% sucrose (Regente and de la Canal, 2000). Controls were performed replacing Helja solution with the same volume of water. After 16 h of incubation at 25 °C and 100% relative humidity the slides were microscopically evaluated for inhibition of spore germination or hyphae growth reduction. The MIC of Helja was defined as the lowest concentration that led to a microscopically visible reduction of hyphal length compared to controls. Three independent biological replicates of antifungal test were performed.

2.4. Viability assays on PDA plates

A *S. sclerotiorum* spore suspension (10^4 cells ml^{-1}) was incubated with protein sample (0.2 µg µl^{-1}) or water as control during 18 h at 4 °C. Subsequently, 10 µl of each treatment were plated in the center of a PDA Petri dish. Antifungal activity was evaluated by observation of mycelial growth and comparing them to controls. The plates were prepared in triplicate and incubated at 25 °C for 7 days.

2.5. Quantitative antifungal activity assays

A quantitative test was performed in 96-well flat microplates containing the protein sample (0.2 µg µl^{-1}) and 1 × 10^4 spores in Sabouraud broth in a final volume of 100 µl (Regente et al., 2014). The fungal growth was monitored by 595 nm optical reading at the indicated times during 3 days of incubation at 25 °C. The percentage of growth inhibition in the presence of Helja was calculated relative to the control in the absence of the lectin, which was considered as 100% growth. At the end of the assay, *S. sclerotiorm* cells were collected from the growth medium and washed in sterile water for further evaluation of morphology and viability. Quantitative tests were performed by triplicate and repeated at least twice. Growth curves were compared using a regression analysis (Infostat/L). The Tukey's 2-tailed *t*-test, assuming unequal variance, was used to determine whether there was a significant difference between two sets of data. *P*-values of 0.05 were considered significant.

2.6. Evans blue and propidium iodide staining

The fungal viability and permeabilization of the plasma membrane were evaluated by Evans Blue and propidium iodide uptake. After evaluation of the microscopic antifungal activity in microslides containing the protein sample (0.05 and 0.2 µg µl^{-1}), Evans Blue dye (Levine et al., 1994) was added to a final concentration of 0.05% and fungal cells were observed by optical microscopy. Following the quantitative antifungal activity test containing the protein sample (0.2 µg µl^{-1}), iodide propidium was added to a final concentration of 50 µg ml^{-1} and the assay was observed under fluorescence microscopy using a Eclipse E200 microscope (Nikon) equipped with an epi-fluorescence unit and a G-2E/C filter set containing an excitation filter at 540/25 nm, a suppressor filter at 630/60 nm and a dichroic mirror at 565 nm (Mansilla et al., 2015).

2.7. Detection of hydrogen peroxide

Detection of hydrogen peroxide in fungal spores was performed by a staining procedure using 3,3′-diaminobenzidine (DAB) (Giudici et al., 2004). Briefly, the fungal spores (1 × 10^5) were incubated on microslides with water (control) or the protein sample (0.05 and 0.2 µg µl^{-1}) in the presence of 0.5 mg ml^{-1} DAB. 2 mg ml^{-1} DAB solution was prepared and one-quarter of the final volume was added to the assay. The slides were microscopically evaluated for production of endogenous H_2O_2 as a brown pellet.

2.8. Covalent conjugation of FITC to Helja and binding to fungal spores

Fluorescein isothiocyanate (FITC) was covalently coupled to Helja