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Plant–pathogen interactions: Sugarcane glycoproteins induce chemotaxis of smut teliospores by cyclic contraction and relaxation of the cytoskeleton

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ABSTRACT

A fraction of active glycoproteins isolated from sugarcane cultivars resistant to smut (*Sporisorium scitamineum*) contains several peptides with different biological activities. One of these proteins is active as a positive chemotactic stimulus on smut teliospores. The displacement of teliospores in a liquid medium to a source containing these glycoproteins describes a Gaussian kinetic both versus time and versus the amount of glycoprotein used as a stimulating agent. Confocal and both transmission and scanning electronic microscopy show that the displacement of teliospores toward glycoprotein source is achieved by consecutive episodes of cell contraction–relaxation in which the cytoskeleton is involved. The chemotactic movement of teliospores is strongly inhibited by phalloidin and latrunculin A, which implies F-actin polymerization and depolymerization cycles, and by blebbistatin, which implies a contractile protein similar to a myosin II, responsible for the contraction–relaxation of the cytoskeleton. Both proteins have been revealed by Western blot. In addition, microtubules seem to be also involved in the process since nocodazole inhibits chemotactic displacement. Fungal teliospores secrete several of their own proteins to the incubation media that increase teliospore germination and cytoagglutination although they are inactive to induce displacement.

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1. Introduction

Smut is one of the major pathologies of sugarcane. It is produced by *Sporisorium scitamineum*, a filamentous fungus that produces significant losses in crops of *Saccharum officinarum*. There are about 1100 species of smut from more than 75 families that attack Angiosperms. They have a dikaryotic intercellular mycelium derived from the conjugation of compatible sporidia. The mycelium invades the entire plant, producing teliospores. Similar to the rest of Ustilaginales, diploid mycelia of *S. scitamineum* is infective, penetrates the host tissues and damages their meristems (Waller, 1970). The visual disease symptom is the presence of a reproductive structure called "whip". The formation of this structure in the apical meristem of the stem implies its destruction. Since the pathogen can use the opened stomata of the host leaves to penetrate in the internal tissues, it is necessary to think that the teliospores deposited at random on the surface of a leaf, far from stomata, should develop a mechanism of displacement toward the way of entry, as suggested by Santiago et al. (2012). For this rationale, it is important to demonstrate the existence of these mechanisms and to study how they can be carried out. Brand and Gow (2012) summarize the current knowledge on spore movement in plant–pathogen interactions. The two most frequently suggested mechanisms are submicroscopical contractions of helically arranged fibrils within the cell walls and the occurrence of motile appendages in zoospores. Other species of pathogenic fungi produce spores that are able of gliding. Gliding is a form of cell movement that differs from crawling or swimming in which it does not rely on any obvious external organ or change in cell shape and it occurs only in the presence of a substrate. When fungal hyphae are the ones responsible to carry out the penetration into the host tissues, thigmotropism induces hyphal growth following the depressions between epidermal cells surrounding stomata.

It has been proposed that resistance to smut largely depends on the ability of sugarcane to prevent spore penetration. Another possibility is that resistance against the pathogen is based on the production of defensive agents (Lloyd and Pillay, 1980) since, according to Abramovitch et al. (2006) basal mechanisms of defense that exist are

Abbreviations: HMMG, High-molecular mass glycoproteins; MH-IIA, Myosin heavy chain IIA; MH-IIB, Myosin heavy chain IIB; ML, Myosin light chain; MMMG, Mid-molecular mass glycoproteins; PAMP, Pathogen-associated molecular pattern; PBS, Phosphate buffered saline solution; PVP, Polyvinylpyrrolidone; RIPA, Radio-immunoprecipitation assay buffer; SEM, Scanning electron microscopy; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, Transmission electron microscopy.

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induced by the pathogen and consist of molecular patterns associated with the pathogenic one (PAMP = pathogen-associated molecular pattern), similar to the innate immunity in animals, which act during the first steps of the infection. The early response of sugarcane to infection involves a large increase of glycoprotein production, which has been defined as macromolecules of medium and high molecular weight (MMMG, mid-molecular mass glycoproteins and HMMG, high-molecular mass glycoproteins). These glycoproteins cause teliospore cytoagglutination, which hinders invasion (Fontaniella et al., 2002). In this view, these glycoproteins would function as factors of sugarcane resistance. Also, HMMG and MMMG decrease the germination rate of teliospores. This could be related to the inhibition of the germinative tube emergence as a result of cytoskeleton disruption and teliospore aggregation state (Fontaniella et al., 2002). In a general way, resistant cultivars to smut produce an amount of these glycoproteins higher than that produced by susceptible ones (Millanes et al., 2005). Studying *Saprolegnia ferax*, Bachewich and Heath (1998) showed that F-actin recruits and stabilizes several cytosolic and membrane factors to enable growth of new hyphae. In addition, Fuchs et al. (2005) show that F-actin is essential to polarized growth during corn infection by *Ustilago maydis*, another closely related smut fungus. Millanes et al. (2005) showed that sugarcane glycoproteins prevented cell polarization of smut teliospores prior to germination and the output of the germinative tube. These glycoproteins mediate their effects through binding to specific receptors in the wall of the teliospores (Legaz et al., 1995, 1998).

HMMG and MMMG include different glycoproteins. Two of them are endowed with arginase and chitinase activities (Millanes et al., 2005), two more develop β-1,3- and β-1,4-glucanase activities whereas another one has tentatively identified as a dirigent protein (submitted). The first enzyme is related to the synthesis of polyamines. In susceptible sugarcane cultivars, plant arginase is braked and retained outside the teliospores by its binding to a specific fungal cell wall receptor (Millanes et al., 2008). In resistant plants, arginase from glycoprotein fraction is internalized by fungal teliospores. The arginase then hydrolyzes arginine to produce ornithine and later putrescine, a diamine that disrupts the cytoskeleton and prevents cell polarity required for germination and development. The mechanism of inhibition of pathogen growth seems to be due, at least in part, to the accumulation of putrescine inside the cell (Millanes et al., 2005). Production of glycoproteins, other than arginase, also occurs in inoculated plants. These act as chemoattractants, favoring the motility of teliospores toward the points of cytoagglutination, in a process mediated by cytoskeleton rearrangement. Thus, teliospores are attracted and aggregated by the action of glycoproteins that act as chemotactic stimulus.

In fungal cells, polarity establishment and the maintenance of cell asymmetry are essential properties that govern morphogenesis, favoring spore germination and causing monopolar filament growth. The cytoskeleton transports vesicles bringing enzymes and precursors required for membrane growth and *de novo* synthesis of the cell wall (Berepiki et al., 2011; Egan et al., 2012). There are three types of cytoskeletal polymers: microfilaments, made of actin; microtubules, made of tubulin; and intermediate filaments, although fungi do not contain these last polymers.

There is a plethora of actin-binding proteins that control filament stability, bundling and cross-linking. From these, the myosin superfamily is one of the most important. Myosins contain ATPase activity, being able to transform the chemical energy of ATP in motion through conformational changes of its structure (Vicente-Manzanares et al., 2009). In general, hydrolysis of ATP binds the myosin head to the actin microfilament, while removal of the free phosphate causes the separation of myosin from actin (Berger et al., 2001).

Actin and myosin are heavily involved in the motility and migration of numerous cell types, e.g. fibroblasts. In these, actin and myosin drive the formation of specific subcelular structures, e.g. filopodia, lamellipodia or stress fibers. It seems that actin may also play a key role in the movement of the fungal cells: the polymerization of actin in the generator limit from the edge of a cell in motion is critical for cell migration. In addition to forming specific structures involved in cell migration, actomyosin also controls cytoplasmic retrograde flow and, together with osmotic force, regulate hydrostatic pressure (Stewart et al., 2011).

In this work, cellular bases of teliospore chemotaxis induced by sugarcane glycoproteins and the involvement of cytoskeleton in the movement were studied.

2. Materials and methods

2.1. Plant material

Teliospores of pathogen *Sporisorium scitamineum* (Syd.) and plants of *Saccharum officinarum* (L.) field grown in "Antonio Mesa" Sugar cane Experimental Station (Jovellanos, Matanzas, Cuba) were used throughout this work. Sugarcane sensitive and resistant cultivars to smut disease (Ba 42–231 cv. and My 55–14 cv., respectively) were employed in the assays in order to verify the differential defensive response to smut disease. Blebbistatin, latrunculin A and nocodazole were from EMD Biosciences (Billerica, MA). All the chemicals, except when indicated, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Purification of HMMG and MMMG sugarcane glycoproteins

Ten stalks of 11-month-old Ba 42–231 cv. and My 55–14 cv. sugar cane plants were cut in segments of approximately 50 cm in length and crushed for three consecutive times in a three-cylinder custom press to extract the juice. Juices were centrifuged at 5000 × *g* for 15 min at 4 °C (Sigma 2k15 centrifuge). The pellets were discarded and the supernatants were filtered through Whatman No. 1 filter paper (Whatman, Maidstone, Kent, UK).

To separate sugarcane glycoproteins, 5 mL of clarified juice was filtered through a Sephadex G-10 (Pfizer-Pharmacia, New York, NY, USA) column (20 cm × 2.5 cm) embedded in 10 mM sodium phosphate buffer, pH 6.8. Elution was also carried out with the same buffer. The void volume (28 mL) was discarded, whereas the following 12 mL contained both HMMG and MMMG glycoproteins. Two filtration processes were required to obtain 24 mL of these glycoproteins, and this volume was concentrated in a Speedvac SPD111V Thermo concentrator (Thermo Fisher Scientific Inc., Rockford, IL, USA). Concentrated fraction (4 mL) was then loaded onto a Sephadex G-50 column (35 cm × 2.5 cm) and eluted in the same way. The void volume (48 mL) was discarded. The next 20 mL contained HMMG and the following 58 mL contained MMMG glycoproteins (Legaz et al., 1995). The final volume was concentrated in a Speedvac SPD111V Thermo concentrator. Eluted fractions were monitored for protein according to the Lowry method (Lowry et al., 1951).

The composition of both HMMG and MMMG has been verified by capillary electrophoresis according to Legaz et al. (1998). They contain six different glycoproteins that have been identified as arginase and chitinase (Millanes et al., 2005), β-1,3- and β-1,4-glucanases, a dirigent protein (submitted) and an unknown protein.

2.3. Purification of arginase from HMMG and preparation of fungal protein from the incubation media

Sugarcane arginase was pre-purified by affinity chromatography using cyanogen bromide-activated agarose bearing urease secreted from *Evernia prunastri* thalli, according to Díaz et al. (2011). About 2.0 g of agarose was fully hydrated with MilliQ water and mixed with 20 mL of a solution of purified urease containing 0.5 mg protein mL^{-1} in 0.1 M phosphate buffer (pH 7.4) for 16 h at 4 °C. Glycine (0.6 g) was added to the mixture and stored at room temperature for 8 h. Beads of activated agarose containing bound urease were packed in

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