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Chitosan enhances parasitism of *Meloidogyne javanica* eggs by the nematophagous fungus *Pochonia chlamydosporia*

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ABSTRACT

Pochonia chlamydosporia (Pc), a nematophagous fungus and root endophyte, uses appressoria and extracellular enzymes, principally proteases, to infect the eggs of plant parasitic nematodes (PPN). Unlike other fungi, Pc is resistant to chitosan, a deacetylated form of chitin, used in agriculture as a biopesticide to control plant pathogens. In the present work, we show that chitosan increases *Meloidogyne javanica* egg parasitism by *P. chlamydosporia*. Using antibodies specific to the Pc enzymes VCP1 (a subtilisin), and SCP1 (a serine carboxypeptidase), we demonstrate chitosan elicitation of the fungal proteases during the parasitic process. Chitosan increases VCP1 immuno-labelling in the cell wall of Pc conidia, hyphal tips of germinating spores, and in appressoria on infected *M. javanica* eggs. These results support the role of proteases in egg parasitism by the fungus and their activation by chitosan. Phylogenetic analysis of the Pc genome reveals a large diversity of subtilisins (S8) and serine carboxypeptidases (S10). The VCP1 group in the S8 tree shows evidence of gene duplication indicating recent adaptations to nutrient sources. Our results demonstrate that chitosan enhances Pc infectivity of nematode eggs through increased proteolytic activities and appressoria formation and might be used to improve the efficacy of *M. javanica* biocontrol.

Introduction

Plant-parasitic nematodes (PPN) are serious pests of all agricultural systems, causing extensive economic losses (Davies & Elling 2015). The genus *Meloidogyne* is notable due to the wide range of crops it parasitizes (Sahebani & Hadavi 2008). Control of PPN is usually through chemical nematicides, but their use has been restricted because of their toxicity, risk to

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the environment, high cost, and low efficacy after repeated applications (Dong & Zhang 2006).

The nematophagous fungus *Pochonia chlamydosporia* (Goddard) Zare & W. Gams is a facultative parasite of nematode eggs predominantly of cyst and root-knot nematodes (Giné *et al.* 2013; Vieira dos Santos *et al.* 2013), with evident potential as a biocontrol agent and sustainable alternative to chemical pesticides for *Meloidogyne* control (Bontempo *et al.* 2014; Viggiano *et al.* 2014). To parasitize PPNs, *P. chlamydosporia* (Pc) needs to adhere to eggs, to differentiate appressoria for penetration (Lopez-Llorca *et al.* 2002), and to excrete extracellular enzymes for eggshell degradation (Yang *et al.* 2013). In nematophagous fungi, the production of these enzymes is directly related to the structure and composition of the eggshell. The egg is the most resistant stage of the life cycle of nematodes since the shell comprises large amounts of the recalcitrant biopolymer chitin in addition to protein (Bird & McClure 1976; Bird & Bird 1991). Proteases and chitinases are therefore considered putative pathogenicity factors (Casas-Flores & Herrera-Estrella, 2007), with subtilisins as key proteinases secreted by Pc (Segers *et al.* 1996) and *Pochonia rubescens* (Lopez-Llorca & Robertson 1992).

The similarities in structure and composition of nematode eggshells and insect cuticles could be responsible for a coevolution of entomopathogenic and nematophagous fungi (Macia-Vicente *et al.* 2011). *Pochonia chlamydosporia* and the closely related insect pathogenic fungus *Metarhizium anisopliae* secrete, as main extracellular proteases, the subtilisins VCP1 and PR1, respectively. They are immunologically related with similar pIs 7–10 and similar molecular weights (~33 kDa) (Segers *et al.* 1995), and they show large similarities in amino acid sequences (Larriba *et al.* 2012). In *Me. anisopliae*, carboxypeptidases were detected when penetrating the host cuticle (St Leger *et al.* 1994; Santi *et al.* 2010), and both Pr1 subtilisin and carboxypeptidase show increased activity in the presence of chitin, a structural component of the insect cuticle.

Unlike *Me. anisopliae*, little is known about the regulation of *P. chlamydosporia* VCP1 during the parasitic process. In previous studies, we identified a *P. chlamydosporia* serine carboxypeptidase, SCP1, which is expressed during endophytic colonisation of barley roots (Lopez-Llorca *et al.* 2010). This protease has been cloned and characterized (Larriba *et al.* 2012), and interrogation of the recently sequenced *P. chlamydosporia* genome shows that the serine protease family is encoded by roughly 190 genes (Larriba *et al.* 2014). In addition, proteomic studies of *P. chlamydosporia* grown using chitin or chitosan as the main nutrient sources have shown that chitosan elicits the expression of a number of proteins including the protease VCP1 (Palma-Guerrero *et al.* 2010).

Chitosan is a linear polysaccharide of randomly distributed β-(1Õ4)-linked D-glucosamine and N-acetyl-β-D-glucosamine obtained by partial de-acetylation of chitin (Dutta *et al.* 2004). It was reported to reduce the number of galls and J2 of the root-knot nematode *Meloidogyne incognita* in soil (Radwan *et al.* 2012) and has been found to increase conidiation of fungal pathogens of invertebrates (FPI) such as entomopathogenic and nematophagous fungi, including *P. chlamydosporia* (Palma-Guerrero *et al.* 2008). However, the effects of chitosan on the infectivity of FPIs such as *P. chlamydosporia* have yet to be determined. Consequently, we investigate here the effect of chitosan on appressorial differentiation, *Meloidogyne javanica* egg parasitism and the production of VCP1 and SCP1 serine proteases by *P. chlamydosporia*. In addition, we use VCP1- and SCP1-specific antibodies to determine the spatio-temporal expression of these enzymes during the parasitic process, and use phylogenetics to determine the relatedness of Pc VCP1 and SCP1 in the S8 and S10 families of proteases.

Materials and methods

Fungal and nematode cultures

The *Pochonia chlamydosporia* used in this work was the isolate Pc123 (ATCC No. MYA-4875; CECT No. 20929) (Olivares-Bernabeu & Lopez-Llorca 2002). The fungus was grown on corn meal agar (CMA) (Becton Dickinson and Company) at 25 °C in the dark. Populations of *Meloidogyne javanica* were kindly provided by Drs. Soledad Verdejo Lucas (IFAPA, Almeria, Spain) and Caridad Ros (IMIDA, Murcia, Spain) and were maintained on susceptible tomato plants (*Solanum lycopersicum* Mill. cv. Marglobe). Nematode egg masses were dissected from RKN-infested roots and kept at 4 °C until used. Egg masses were hand-picked and surface-sterilized as described previously (Escudero and Lopez-Llorca 2012).

Preparation of chitosan

Chitosan with a de-acetylation degree of 80.6 % and a molecular weight of 70 kDa, was obtained from Marine BioProducts GmbH (Bremerhaven, Germany) and was prepared as described previously (Palma-Guerrero *et al.* 2008). Briefly, chitosan was dissolved in 0.25 mol l^{-1} HCl and the pH adjusted to 5.6 with NaOH. The resulting solution was dialysed for salt removal with distilled water and autoclaved at 120 °C for 20 min.

Effect of chitosan on appressorium development

Conidia were collected from 2-week-old cultures of *P. chlamydosporia* growing on CMA. They were harvested with 3 ml sterile distilled water and passed through Miracloth (Calbiochem) to remove hyphae. Conidial suspensions (10^6 conidia ml^{-1}) were incubated for 16 h (~80 % germination) at 25 °C in 0.0125 % (w/v) yeast extract in water (YEM) as described previously (St Leger *et al.* 1989). Germlings were then centrifuged at 11 180 *g* for 5 min and supernatants discarded.

Germlings (10^6 germlings ml^{-1}) were incubated with 0, 0.005, 0.01, 0.1, 1, or 2 mg ml^{-1} chitosan in 0.0125 % YEM and placed on 1 cm × 1 cm polyvinyl chloride (PVC) squares to induce appressorium differentiation (Lopez-Llorca *et al.* 2002). After 10 h, squares were examined microscopically with an Olympus BH-2 light microscope at 400×. Approximately 60 germlings were analysed for appressorial differentiation in a total of five fields per treatment (chitosan concentration). The experiment was carried out twice.

Effect of chitosan on egg-infection

Egg-infection bioassays were carried out using ten-well microscope slides (Waldemar Knittel). Each well contained 20 µl